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(54) Title: METHODS AND COMPOSITIONS FOR MODULATING AN IMMUNE RESPONSE

(57) Abstract

The present invention features new approaches for modulating immune responses. In particular, the invention features methods for modulating type 1 immune responses in a subject using modulators of Eta–1(early T lymphocyte activation–1)/osteopontin. Examplary methods feature methods of treating infections, immune disorders and diseases, autoimmune disorders and diseases, various immunodeficiencies and cancer. Also provided are biosynthetic immunomodulatory molecules that include functional domains derived from Eta–1/osteopontin. Preferred biosynthetic immunomodulatory molecules include an IL–12 stimulatory domain derived from Eta–1/osteopontin or an IL–10 inhibitory domain derived from Eta–1/osteopontin. The immunomodulatory molecules of the present invention are capable of biasing an immune response in a subject towards a type 1 immune response. Accordingly, therapeutic uses are disclosed which are based on the biosynthetic immunomodulatory molecules of the present invention.

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METHODS AND COMPOSITIONS FOR MODULATING AN IMMUNE RESPONSE

Related Applications

This application claims the benefit of previously filed U.S. Provisional Application Serial No. 60/129,772, filed April 15, 1999, the content of which is hereby incorporated by reference. This invention was made with government support from the National Institutes of Health. Accordingly, the government may have certain rights in the invention.

Background of the Invention

Efficient development of inflammatory responses and protection against most infectious pathogens depends, in part, on monocytes as the final effector cells. The participation of monocytes in inflammation entails emigration of these cells from peripheral blood into infected tissues, where they produce cytokines that regulate diverse processes including anti-microbial activity, cell growth, differentiation and wound healing (Singer *et al.* (1995) *J. Clin. Invest.* 95:2178-2186. In acute reactions, monocytes may be attracted by neutrophils whereas, in delayed responses, they act in a neutrophil-independent manner. Secretion of T-cell cytokines plays a pivotal role in recruitment of monocytes to sites of infection and activation of these emigrant cells to express bacteriocidal activity. The mechanism of this process bears intensely on wound healing and delayed-type immune responses but its molecular basis is not understood.

An important component of this T-cell dependent response is a protein known as Eta-1 (for early T lymphocyte activation-1)/osteopontin, which mediates macrophage chemotaxis *in vitro* (Weber *et al.* (1996) *Science* 271:509-512, recruits monocytes to inflammatory sites *in vivo* (Singh *et al.* (1990) *J. Exp. Med.* 171:1931-1942) and regulates immunological resistance to several intracellular pathogens (Patarca *et al.* (1989) *J. Exp. Med.* 170:145-161; Lampe *et al.* (1991) *J. Immunol.* 147:2902-2906. Inbred mouse strains that carry an allele of Eta-1/osteopontin which allows high level expression in activated T-cells are resistant to lethal effects of infection by the intracellular parasite *Rickettsia tsutsugamushi* while inbred strains carrying a low expression allele do not develop a population of bacteriocidal monocytic migrants at the

area of infection and succumb to systemic bacteremia (Patarca *et al.* (1990) *Immunol. Rev.* (116:1-16). Eta-1/osteopontin expression has also been linked to granuloma formation, where it may regulate the chronic cellular response associated with tuberculosis infection and silicosis (Nau *et al.* (1997) *Proc. Natl. Acad. Sci. USA* 94:6414-6419. Moreover, in experimental glomerulonephritis, neutralizing antibodies to osteopontin greatly diminish the influx of macrophages and T-cells and reduce kidney damage (Yu *et al.* (1998) *Proc. Am. Assoc. Physicians* 110:50-64). While Eta-1/ostepontin has been implicated in at least certain immunological reactions, its precise role in the immune system has not previously been established. Moreover, Eta-1 is a multifunctional protein having diverse biological roles including , but not limited to, bone resorption, neoplastic transformation, atheromatous plaque formation, dystrophic calcification of inflamed and/or damaged tissues and resistance to certain bacterial infections. (See *e.g.*, Oldberg *et al.* (1986) *Proc. Natl. Acad. Sci. USA* 83:8819; Ross *et al.* (1993) *J. Biol. Chem.* 268:9901; Giachelli *et al.* (1995) *Ann. NY Acad. Sci.* 760:109; Senger *et al.* (1983) *Nature* 302:714; and Srivatsa *at al.* (1997) *J. Clin. Invest.* 99:996). With regards in particular, to understanding the role of Eta-1/osteopontin in immunity, there exists a need to understand the precise role that Eta-1/osteopontin plays in regulating immune responses in order to develop new approaches to treating immune disorders and diseases.

Summary of the Invention

The present invention establishes that Eta-1/osteopontin is a critical regulator of type-1 (cell-mediated) immunity and that this molecule includes a domain that promotes the production of the type 1 cytokine IL-12 and a domain that inhibits the production of the type 2 cytokine IL-10. Thus, the invention provides for the use of Eta-1/osteopontin modulatory agents (i.e, agents that stimulate or inhibit Eta-1/osteopontin activity) to bias an immune response either toward type 1 or type 2 immunity, depending on the clinical situation. The present invention identifies Eta-1/osteopontin as a critical cytokine in type 1 immune responses, in particular, in delayed type hypersensitivity responses. The invention defines Eta-1/osteopontin as a multifunctional molecule which acts as both a stimulator of IL-12 secretion by macrophages and an inhibitor of IL-10 expression. As such, Eta-1/osteopontin serves to bias an organism's cytokine pattern

towards that of a type 1 immune response. In particular, induction of IL-12 and inhibition of IL-10 reflect differential engagement of macrophage receptors: a phosphorylation-dependent interaction between the N-terminal portion of Eta-1/osteopontin and its integrin receptor on macrophages leads to IL-12 expression, while a phosphorylation-independent interaction of a C-terminal domain of Eta-1/osteopontin with CD44 mediates IL-10 inhibition. Moreover, cleavage of Eta-1/osteopontin by thrombin results in a C-terminal fragment of the cytokine which interacts with CD44 and induces macrophage chemotaxis, while engagement of integrin receptors by a non-overlapping N-terminal fragment leads to macrophage spreading and activation.

Based, at least in part, on a detailed understanding of the role this multifunctional cytokine plays in type 1 immune responses, the present invention features novel approaches to modulating immune responses, in particular, in potentiating type 1 immune responses. The invention further features new methods of treating disorders that may benefit from either a type 1 or type 2 immune response. More specifically, the identification of Eta-1/osteopontin as a critical regulator of type 1 immunity allows for selective manipulation of T cell subsets in a variety of clinical situations using the modulatory methods of the invention. The stimulatory methods of the invention (*i.e.*, methods that use an Eta-1/osteopontin stimulatory agent) upregulate the production of the Th1-associated cytokine IL-12 and/or dowregulate the production of the Th2-associated cytokine IL-10, with concomitant promotion of a type 1 immune responses and downregulation of type 2 immune responses. These stimulatory methods that promote a type 1 response can be used, for example, in the treatment of infections (e.g., bacterial, viral), cancer, allergy, burn-associated sepsis and immunodeficiency disorders. In contrast, the inhibitory methods of the invention (*i.e.*, methods that use an Eta-1/osteopontin inhibitory agent) downregulate the production of the Th1-associated cytokine IL-12 and/or upregulate the production of the Th2-associated cytokine IL-10, with concomitant downregulation of a type 1 immune responses and promotion of type 2 immune responses. These inhibitory methods that promote type 2 responses can be used, for example, in the treatment of autoimmune disorders, transplant rejection, granulomatous disorders, herpes simplex keratitis and bacterial arthritis.

Moreover, based on a detailed understanding of the functional domains of Eta-1/osteopontin, the present invention provides biosynthetic molecules which mimic distinct functions of Eta-1/osteopontin for use in a variety of therapeutic applications, in particular, in wound healing, enhancement of the immune response and in treatment of granulomatous disease. In particular, the biosynthetic molecules of the present invention are useful in biasing an immune response towards a delayed type hypersensitivity response, *i.e.*, towards type 1 immunity. A preferred IL-12 stimulatory domain of Eta-1/osteopontin comprises amino acids 71-168 of SEQ ID NO: 2. A preferred IL-10 inhibitory domain of Eta-1/osteopontin comprises amino acids 169-266 of SEQ ID NO: 2. Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

Brief Description of the Drawings

Figure 1A-C demonstrates gramuloma formation in normal, cytokine-deficient and Eta-1/osteopontin-deficient mice. *Figure 1A* depicts the data as the mean number of granulomas per high-power field (HPF) (X200 magnification), mean number of cells per granuloma, and as the product of these two indices, termed "granuloma burden". (Error bars indicate 1 SEM.). *Figure 1B* depicts an analysis of surface antigens expressed by cells within granulomas in the indicated mouse strains. *Figure 1C* depicts cytokine expression by cells from lymph nodes draining the site of granulomas. Data are representative of three independent experiments.

Figure 2A-E demonstrates HSV-1-specific delayed-type hypersensitivity (DHT) reactions in normal and Eta-1/opn$^{-/-}$ mice. *Figure 2A* depicts footpad swelling in Eta-1/opn$^{-/-}$ vs. Eta-1/opn$^{+/+}$ mice inoculated with HSV-1. The right (control, □) and left (HSV-1, ■) footpads of each mouse were measured 24h later using a micrometer. Each data point represents the mean and standard error of three mice/group. *Figure 2B* depicts inhibition of the HSV-1 DHT response in Eta-1/opn$^{+/+}$ mice by acute depletion of Eta-1/opn. *Figure 2C* depicts HSK in Eta-1/opn$^{-/-}$ (open circles) vs. Eta-1/opn$^{+/+}$ (closed circles) mice inoculated with HSV-1. *Figure 2D* depicts HSK in BALB/cBγJ (open circles), Eta-1/opn$^{-/-}$ (closed circles), Eta-1/opn$^{+/+}$ (open squares), and CB-17 (closes squares) mice inoculated with HSV-1. *Figure2E* depicts the cytokine response after

restimulation of draining lymph node cells (from mice 15 days after HSV-1 infection in vivo) or splenic macrophages.

Figure 3A-D demonstrates the differential regulation of macrophage IL-12 and IL-10 responses by purified Eta-1/opn. *Figure 3A* depicts the dose-dependent induction of IL-12 secretion, but not IL-10 secretion, from macrophages by Eta-1/opn. Assays were performed in quadruplets and each data point represents the mean and standard error of two independent experiments. *Figure 3B* depicts selective IL-12 secretion as stimulated by Eta-1/opn as compared to LPS and/or IL-4 which stimulate both IL-12 and IL-10 secretion. Assays were performed in quadruplets and each data point represents the mean and standard error of two independent experiments. *Figure 3C* demonstrates that the inhibitory effects of Eta-1/opn on IL-4-induced IL-10 production by macrophage are unaffected by the presence of anti-IL-12 antibody. Assays were performed in quadruplets and each point represents the mean and standard error of two independent experiments. *Figure 3D* depicts the inhibitory effect of Eta-1 on LPS-activated macrophage IL-10 production. Assays were performed in quadruplets, and each point represents the mean and standard error (error bars) or two independent experiments.

Figure 4 depicts the attachment and spreading of MH-S macrophages on phosphorylated Eta-1/opn, Eta-1/opn, Eta-1/opn-N-terminal fragment, and Eta-1/opn-C-terminal fragment. *Figure 4A* depicts the attachment and spreading of MH-S cells on phosphorylated Eta-1/opn, Eta-1/opn, Eta-1/opn-NT and Eta-1/opn-CT in the presence or absence of the peptide GRGDS (SEQ ID NO:11). *Figure 4B* depicts the correlation between PI-3K activation and spreading of cells on Eta-1/opn, dephosphorylated Eta-1/opn, C- or N-terminal fragment, or NK10 fragment.

Figure 5A-C demonstrates that induction of IL-12 and inhibition of IL-10 occur *via* distinct receptors on macrophages. *Figure 5A* demonstrates that secretion of IL-12 by macrophages is mediated by a 10 kD (NK10) peptide derived from the N-terminal fragment of Eta-1/opn (NT) and is inhibited by a blocking anti-integrin $ß_3$ antibody but is unaffected by antibody to CD44. *Figure 5B* demonstrates that Eta-1/opn-dependent inhibition of IL-4-induced Il-10 production is reversed by anti-CD44 but not by anti-integrin antibodies. *Figure 5C* demonstrates that macrophages from $CD44^{-/-}$ mice are resistant to OEta-1/opn inhibition of the IL-10 response as compared to control mice in

which Eta-1/opn inhibits IL-4 induced IL-10 production. In all panels, mean values and standard errors from at least four data points are shown.

Figure 6A-B demonstrates that that phosphorylation of Eta-1/opn is necessary for engagement of integrin receptors leading to IL-12 production but not for ligation of CD44 leading to IL-10 inhibition by macrophages. *Figure 6A* depicts IL-12 secretion resulting from phosphorylated vs. unphosphorylated Eta-1/opn. *Figure 6B* demonstrates that dephosphorylation of native Eta-1/opn results in loss of IL-12 inducing activity, while phosphorylation of (inactive) recombinant Eta-1/opn restores this function.

Figure 7 is a bar graph demonstrating that ligation of integrin receptors on macrophages (*e.g.*, *via* Eta-1/osteopontin, recombinant phosphorylated Eta-1/osteopontin, N terminal fragment or NK10) causes predominantly IL-12, TGFβ, and TNFα secretion.

Figure 8 is a bar graph demonstrating that ligation of integrin receptors on macrophages causes predominantly IL-12, TGFβ, and TNFα secretion and depicts the effect of various inhibitors (*e.g.*, wortmanin, genestein, chelerythine, pertussis toxin, cytochalasin D, and N-(2-metylpiperazyl)-5-isoquinolinesulfoamide(H-7)) on the cytokine secretion profile. The data are represented as cytokine concentration in media harvested from appropriately treated cells.

Figure 9 is a bar graph representing the data of Figure 8 as a fold-induction of cytokine secreted.

Figure 10 is a schematic diagram of a biosynthetic immunomodulatory molecule of the present invention, termed "immunomodulin-2".

Figure 11 is a schematic diagram of a biosynthetic immunomodulatory molecule of the present invention, termed "immunomodulin-1".

Figure 12 is a bar graph depicting the effect of the biosynthetic immunomodulatory molecules immunomodulin-1 and immunomodulin-2 on IL-12 and IL-10 secretion by macrophages. Data are represented as cytokine concentration in media harvested from appropriately treated cells.

Figure 13 is a bar graph depicting the effect of the biosynthetic immunomodulatory molecules immunomodulin-1 and immunomodulin-2 on IL-12 and IL-10 secretion by macrophages stimulated with IL-4 or LPS. Data are represented as cytokine concentration in media harvested from appropriately treated cells.

Figure 14 is a bar graph depicting the effect of immunomodulin-2 administration in an *in vivo* model of allergy.

Detailed Description of the Invention

The present invention is based, at least in part, on the elucidation of a new role for Eta-1/osteopontin in regulating immune responses, in particular, as a modulator of type-1 immunity. It has been discovered that Eta-1/osteopontin plays a dual role in activation of, for example, the type-1 cytokine IL-12, as well as in the inhibition of the type-2 cytokine IL-10. As such, Eta-1/osteopontin is capable of biasing an immune response in favor of a type-1 response, or a cellular immune response, as compared with a type-2 response, or humoral response. It has been further discovered that the IL-12 stimulatory and IL-10 inhibitory functions of Eta-1/osteopontin can be localized to a specific domains of the naturally-occurring protein. Identification of these biologically active domains of Eta-1/osteopontin has led to the development of new approaches to and therapeutics useful for the treatment of various immune response-related diseases and disorders. Moreover, the role of Eta-1/osteopontin in processes including monocyte recruitment, adhesion and activation (*i.e.*, cytokine secretion) has been analyzed in detail and new mechanisms for performing such functions have been disclosed.

In one aspect, the invention features methods of modulating immune responses, in particular, methods of modulating type-1 immune responses in a subject *or* patient (e.g., a human subject *or patient*) which involve administering to the subject *or* patient an Eta-1/osteopontin modulator such that the immune response (e.g., the type-1 immune response) is modulated. In one embodiment, the Eta-1/osteopontin modulator stimulates Eta-1/osteopontin activity and the type-1 immune response is potentiated. In another embodiment, the Eta-1/osteopontin modulator inhibits Eta-1/osteopontin activity and the type-1 immune response is downregulated. In another embodiment, the Eta-1/osteopontin modulator is administered in a therapeutically effective amount. In another embodiment, the method also includes monitoring the type-1 response in the

subject (*e.g.*, determining the level of a detectable indicator of the type-1 response) and/or comparing the level of the detectable indicator to a control.

In another embodiment, the invention features methods of potentiating type-1 immune responses in a subject or patient that include selecting a patient or subject suffering from a disease or disorder that would benefit from a potentiated type-1 immune response (*e.g.*, selecting an individual patient or subject from the human population) and administering to that patient an Eta-1/osteopontin stimulatory modulator such that the type-1 immune response is potentiated. In a preferred embodiment, the disease or disorder that would benefit from a potentiated type-1 immune response is at least one of the following: (1) burn-associated sepsis, (2) bacterial infection, (3) viral infection, (4) cancer, (5) immunodeficiency disorders, (6) AIDS, (7) bone marrow transplant-related immunodeficiency, (7) chemotherapy-related immunodeficiency and (7) allergy.

In another embodiment, the invention features methods of downregulating type-1 immune responses in a subject or patient that include selecting a patient or subject suffering from a disorder that would benefit from a downregulated type-1 immune response (*e.g.*, selecting an individual patient or subject from the human population) and administering to the patient or subject an Eta-1/osteopontin inhibitory modulator such that the type-1 immune response is downregulated. In a preferred embodiment, the disease or disorder that would benefit from a downregulated type-1 immune response is at least one of the following: (1) bacterial arthritis, (2) granulomatous disorder, (3) herpes simplex keratitis, and (4) autoimmune diseases.

In yet another embodiment, the present invention features methods for enhancing production of a type-1 immune response-associated cytokine (*e.g*, interleukin-2 (IL-2), interleukin-12 (IL-12) and/or interferon-γ (IFN-γ)) by an immune cell (*e.g.*, a human immune cell) that include contacting the cell with an Eta-1/osteopontin stimulatory modulator such that production of the cytokine is enhanced. In yet another embodiment, the invention features methods for downregulating production of a type-2 immune response-associated cytokine (*e.g.*, interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6), and/or interleukin-10 (IL-10)) by an immune cell that include contacting (*e.g.*, *in vivo* or *in vitro*) the cell with an Eta-1/osteopontin inhibitory modulator such that production of the cytokine is downregulated. Exemplary

immune cells include macrophages, dendritic cells, T cells, B cells, monocytes and/or neutrophils. In yet another embodiment, the invention features methods for stimulating interleukin-12 (IL-12) production by macrophages that include contacting the macrophages with an Eta-1/osteopontin stimulatory modulator such that production of IL-12 is stimulated. In yet another embodiment, the invention features a method for inhibiting interleukin-10 (IL-10) production by macrophages that includes contacting the macrophages with an Eta-1/osteopontin stimulatory modulator such that production of IL-10 is inhibited.

The present invention also features methods for potentiating type-1 immune responses in a subject or patient that include culturing immune effector cells isolated from the subject or patient in the presence of an Eta-1/osteopontin stimulatory modulator and administering the cultured cells to the subject such that the type-1 immune response in the subject is potentiated. Also featured are modified tumor cells, for example, irradiated tumor cells transduced with Eta-1/osteopontin and such modified tumor cells further transduced with GMCSF.

Preferred Eta-1/osteopontin modulators of the present invention include, but are not limited to, isolated Eta-1/osteopontin polypeptides and biologically active fragments thereof, isolated nucleic acid molecules that encodes Eta-1/osteopontin polypeptides and that encode biologically active fragments thereof. In one embodiment, the Eta-1/osteopontin modulator is an Eta-1/osteopontin polypeptide at least 90% identical to a polypeptide having the amino acid sequence of SEQ ID NO:2. In another embodiment, the Eta-1/osteopontin modulator is an Eta-1/osteopontin polypeptide having the amino acid sequence of SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6.

In another embodiment, the Eta-1/osteopontin modulator is an isolated nucleic acid molecule at least 90% identical to a nucleic acid molecule having the nucleotide sequence of SEQ ID NO:1. In yet another embodiment, the Eta-1/osteopontin modulator is an isolated nucleic acid molecule having the nucleotide sequence of SEQ ID NO:1, SEQ ID NO:3 or SEQ ID NO:5.

In another embodiment, the Eta-1/osteopontin modulator is a biologically active fragment of Eta-1/osteopontin, or a nucleic acid molecule encoding such a biologically active fragment. Preferred biologically active fragments include IL-12 stimulatory domains and/or IL-10 inhibitory domains of Eta-1/osteopontin. In one

embodiment, an IL-12 stimulatory domain includes an amino acid sequence between 65 and 160 amino acids in length and is at least 90% identical to amino acids 71 to 168 of SEQ ID NO:2. In another embodiment, an IL-10 inhibitory domain includes an amino acid sequence between 65 and 160 amino acids in length and is at least 90% identical to amino acids 169-266 of SEQ ID NO:2.

Additional preferred Eta-1/osteopontin modulators of the present invention include compounds that specifically bind to Eta-1/osteopontin polypeptides, compounds that specifically binds to Eta-1/osteopontin target molecules, compounds that specifically modulate the activity of Eta-1/osteopontin polypeptides and compounds that specifically modulate the activity of Eta-1/osteopontin target molecules. In one embodiment, the Eta-1/osteopontin modulator is an antibody that specifically binds to Eta-1/osteopontin. In another embodiment, the Eta-1/osteopontin modulator is an antibody that specifically binds an Eta-1/osteopontin target molecule (*e.g.*, an antibody that specifically binds to an integrin or a CD44 molecule).

In yet another embodiment, the Eta-1/osteopontin modulator is a biosynthetic immunomodulatory molecule. Preferred biosynthetic immunomodulatory molecules include an IL-12 stimulatory component (*e.g.*, an IL-12 stimulatory component derived from Eta-1/osteopontin) and a biomodular component, forming a molecule which modulates an immune response. For example, an IL-12 stimulatory component can be an Eta-1/osteopontin-derived polypeptide (*e.g.*, a polypeptide that has an amino acid sequence between 65 and 160 amino acid residues in length and is at least 90% identical to amino acids 71 to 168 of SEQ ID NO:2).

Additional preferred biosynthetic immunomodulatory molecules include an IL-10 inhibitory component (*e.g.*, an IL-10 inhibitory component derived from Eta-1/osteopontin) and a biomodular component, forming a molecule which modulates an immune response. For example, an IL-10 inhibitory component can be an Eta-1/osteopontin-derived polypeptide (*e.g.*, a polypeptide that has between 65 and 160 amino acid residues in length and is at least 90% identical to amino acids 169 to 266 of SEQ ID NO:2). Exemplary biomodular component include signal peptides, calcium/apatite binding domains and/or heparin binding domains. Additional preferred biosynthetic immunomodulatory molecules include at least two biomodular components.

A preferred biosynthetic immunomodulatory molecule includes [Picture claim for Immunomodulin-2] (*e.g.*, the biosynthetic immunomodulatory molecule comprising the amino acid sequence of SEQ ID NO:8 and/or the molecule encoded by nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO:7). Another preferred biosynthetic immunomodulatory molecule includes an IL-10 inhibitory component, a signal peptide, a calcium/apatite binding domain and a heparin binding domain (*e.g.*, the biosynthetic immunomodulatory molecule comprising the amino acid sequence of SEQ ID NO:10 and/or the molecule encoded by nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO:9).

Preferred biosynthetic immunomodulatory molecules of the present invention are useful for modulating an immune response (*e.g.*, in a subject or patient, for example, a human subject or patient) for example, in modulating cytokine secretion, regulation of chemotaxis, regulation of hapotaxis, and regulation of cell spreading. Also featured are isolated nucleic acid molecules that encode the biosynthetic immunomodulatory molecules of the present invention, expression vectors that include such nucleic acid molecules, and host cell including such vectors. The present invention also features methods of producing biosynthetic immunomodulatory molecule that include culturing such host cell under conditions such that the immunomodulatory molecule is produced. Pharmaceutical composition that include the biosynthetic immunomodulatory molecules of the present invention are also featured.

The present invention also features method of modulating an immune response in a cell that include contacting the cell with a featured biosynthetic immunomodulatory molecule such that an immune response is modulated. In a preferred embodiment the cell is present within a subject or patient and the immunomodulatory molecule is administered to the subject.

In order that the present invention may be more readily understood, certain terms are first defined herein.

The term "Eta-1/osteopontin" refers to a protein known in the art and referred to herein interchangeably as "early T lymphocyte activation-1", "Eta-1", "osteopontin", "opn" and "Eta-1/opn". Eta-1/osteopontin was originally identified in bone

and is now also known to be secreted T cells early during their activation by a variety of stimuli. Eta-1/osteopontin is a noncollagenous adhesive matrix protein normally found in bone and at epithelial cell surfaces. Eta-1/osteopontin contains an arginine-glycine-aspartate (RGD)-binding motif common to many extracellular matrix proteins. Eta-1/osteopontin also contains a thrombin cleavage site, cleavage of which alters the proteins adhesive properties. Eta-1 has at least two cellular receptors including integrin and CD44 (Weber *et al.* (1996) *Science* 271:509-512). As described herein, Eta-1 has muliple biological functions. In particular, Eta-1/osteopontin can function as an immune response modulator. (See *e.g.*, U.S. Patent No. 5,049,659 and WO 98/08379). A preferred biological function of Eta-1, as described herein, is in potentiating a type 1 immune response. For a detailed review of the structure and biological functions of Eta-1/osteopontin, see *e.g.*, Denhardt and Guo (1993) 7:1475-1482 and Patarca *et al.* (1993) *Crit. Revs. Imunol.* 13:225-246, and the referenced cites therein.

The term "immune response" includes any response associated with immunity including, but not limited to, increases or decreases in cytokine expression, production or secretion (*e.g.*, IL-12, IL-10, TGFβ or TNFα expression, production or secretion), cytotoxicity, immune cell migration, antibody production and/or immune cellular responses. The phrase "modulating an immune response" or "modulation of an immune response" includes upregulation, potentiating, stimulating, enhancing or increasing an immune response, as defined herein. For example, an immune response can be upregulated, enhanced, stimulated or increased directly by use of a modulator of the present invention (*e.g.*, a stimulatory modulator). Alternatively, a modulator can be used to "potentiate" an immune response, for example, by enhancing, stimulating or increasing immune responsiveness to a stimulatory modulator. The phrase "modulating an immune response" or "modulation of an immune response" also includes downregulation, inhibition or decreasing an immune response as defined herein. Immune responses in a subject or patient can be further characterized as being either type-1 or type-2 immune responses.

A "type-1 immune response", also referred to herein as a "type-1 response" or a "T helper type 1 (Th1) response" includes a response by $CD4^+$ T cells that is characterized by the expression, production or secretion of one or more type-1 cytokines and that is associated with delayed type hypersensitivity responses. The

phrase "type-1 cytokine" includes a cytokine that is preferentially or exclusively expressed, produced or secreted by a Th1 cell, that favors development of Th1 cells and/or that potentiates, enhances or otherwise mediates delayed type hypersensitivity reactions. Preferred type-1 cytokines include, but are not limited to, interleukin-2 (IL-2), interleukin-12 (IL-12), interferon-γ (IFN-γ) and tumor necrosis factor-β (TNF-β).

A "type-2 immune response", also referred to herein as a "type-2 response or a "T helper type 2 (Th2) response" refers to a response by $CD4^+$ T cells that is characterized by the production of one or more type-2 cytokines and that is associated with humoral or antibody-mediated immunity (*e.g.*, efficient B cell, "help" provided by Th2 cells, for example, leading to enhanced IgG1 and/or IgE production). The phrase "type-2 cytokine" includes a cytokine that is preferentially or exclusively expressed, produced or secreted by a Th2 cell, that favors development of Th2 cells and/or that potentiates, enhances or otherwise mediates antibody production by B lymphocytes. Preferred type-2 cytokines include, but are not limited to, interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6), interleukin-10 (IL-10) and interleukin-13 (IL-13).

Cytokine expression, secretion or production modulates or further enhances or upregulates an immune response, for example, a type-1 of type-2 immune response. For example, it is known that cytokines play a dominant role in controlling the differentiation of T helper precursors (Th0) to either the Th1 or Th2 lineage. Type-1 cytokines, such as IFN-γ, can enhance the development of Th1 cells and inhibit the development of Th2 cells, whereas type-2 cytokines, such as IL-4 and IL-10, can enhance the development of Th2 cells and inhibit the development of Th1 cells. Thus, cytokines can reciprocally regulate the development and/or progression of either a type-1 or a type-2 response.

Cytokine expression, secretion or production can also be an indicator of an immune response, for example, an indicator of a type-1 or type-2 immune response.

For example, a "cytokine profile" can be indicative of a type-1 or type-2 immune response. The term "cytokine profile" includes expression, production or secretion of at least one cytokine associated with a particular type of immune response (*e.g.*, a type-1 or type-2 immune response) and/or includes diminished or reduced expression, production or secretion of at least one cytokine associated with a mutually

exclusive type of immune response (*e.g.*, a type-2 or type-1 immune response, respectively). For example, a type-1 cytokine profile can include enhanced or increased expression, production or secretion of at least one of interleukin-2 (IL-2), interleukin-12 (IL-12), interferon-γ (IFN-γ) and tumor necrosis factor-β (TNF-β) and/or can include reduced or decreased expression, production or secretion of at least one of interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6) and interleukin-10 (IL-10). Likewise, a type-2 cytokine profile can include expression, production or secretion of at least one of interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6) and interleukin-10 (IL-10) and/or can include reduced or decreased expression, production or secretion of at least one of interleukin-2 (IL-2), interleukin-12 (IL-12), interferon-γ (IFN-γ) and tumor necrosis factor-β (TNF-β).

The phrase "type-1 immunity" includes immunity characterized predominantly by type-1 immune responses (*e.g.*, delayed type hypersensitivity, macrophage activation and or cellular cytotoxicity), by expression, production or secretion of at least one type-1 cytokine and/or expression of a type-1 immunity cytokine profile. The phrase "type-2 immunity" includes immunity characterized predominantly by type-2 immune responses (*e.g.*, B cell help, IgG1 and/or IgE production, eosinophil activation, mast cell stimulation and/or macrophage deactivation), by expression, production or secretion of at least one type-2 cytokine and/or expression of a type-2 immunity cytokine profile.

The course of certain disease states is influenced by whether a predominant type-1 or type-2 response is mounted. For example, in experimental leishmania infections in mice, animals that are resistant to infection mount predominantly a type-1 immune response, whereas animals that are susceptible to progressive infection mount predominantly a type-2 immune response (Heinzel *et al.* (1989) *J. Exp. Med.* 169:59-72; and Locksley and Scott (1992) *Immunoparasitology Today* 1:A58-A61). In murine schistosomiasis, a switch from type-1 to type-2 immunity is observed coincident with the release of eggs into the tissues by female parasites and is associated with a worsening of the disease condition (Pearce *et al.* (1991) *J. Exp. Med.* 173:159-166; Grzych *et al.* (1991) *J. Immunol.* 141:1322-1327; and Kullberg *et al.* (1992) *J. Immunol.* 148:3264-3270). Many human diseases, including chronic infections (such as with human immunodeficiency virus (HIV) or tuberculosis) and

certain metastatic carcinomas, also are characterized by a type-1 to type-2 switch. (see *e.g.*, Shearer and Clerici (1992) *Prog. Chem. Immunol.* 54:21-43; Clerici and Shearer (1993) *Immunol. Today* 14:107-111; Yamamura *et al.* (1993) *J. Clin. Invest.* 91:1005-1010; Pisa *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:7708-7712; Fauci (1988) *Science* 239:617-623). Furthermore, certain autoimmune diseases have been shown to be associated with a predominant type-1 response. For example, patients with rheumatoid arthritis have predominantly Th1 cells in synovial tissue (Simon *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:8562-8566) and experimental autoimmune encephalomyelitis (EAE) can be induced by autoreactive Th1 cells (Kuchroo *et al.* (1993) *J. Immunol.* 151:4371-4381).

The phrase "potentiating or potentiation of a type-1 or type-2 immune response" includes upregulation, stimulation or enhancement of a type-1 or type-2 response, respectively (*e.g.*, commitment of T helper precursors to either a Th1 or Th2 lineage, further differentiation of cells to either the Th1 or Th2 phenotype and/or continued function of Th1 or Th2 cells during an ongoing immune response). For a review of Th1 and Th2 subsets see, for example, Seder and Paul (1994) *Ann. Rev. Immunol.* 12:635-673.

The phrase "potentiating or potentiation of a type-1 immune response" also includes downregulation or inhibition of a type-2 immune response. The phrase "potentiating or potentiation of a type-2 immune response" also includes downregulation or inhibition of a type-1 immune response.

The term "immunomodulatory molecule", used interchangeably herein with the term "immunomodulatory "agent" includes a molecule or agent which has a modulatory or regulatory activity which is normally associated with an immune response in an organism, for example, higher animals and humans. An activity (*e.g.*, a biological or functional activity) associated with an immune response can be any activity associated with resistance of the organism to infection with microorganisms, response to infection or response to disease. The term "activity", "biological activity" or "functional activity", refers to an activity exerted by a molecule of the invention (*e.g.*, a immunomodulatory molecule, for example, a protein, polypeptide, fragment, nucleic acid molecule, antibody, biosynthetic immunomodulatory molecule, or the like) as

determined *in vivo*, or *in vitro*, according to standard techniques and/or methods such as those described in the Examples.

The term "immune cell" includes cells of the immune system which are capable of expressing, producing or secreting cytokines that regulate an immune response, for example a type-1 or type-2 immune response. Preferred immune cells include human immune cells. Exemplary preferred immune cells include, but are not limited to, macrophages, dendritic cells, T cells, B cells and neutrophils. Immune cells are also referred to herein as "immune effector cells". The term "macrophage" includes all cells within the macrophage lineage, including monocytes, circulating macrophages, tissue macrophages, activated macrophages, and the like, from mammals (*e.g.*, from humans). The term "T cell" (*i.e.*, T lymphocyte) is intended to include all cells within the T cell lineage, including thymocytes, immature T cells, mature T cells and the like, from mammals (*e.g.*, from humans).

The phrase "contacting" (*e.g.*, contacting a cell, for example, with an agent or modulator) is intended to include incubating the agent and the cell together *in vitro* (*e.g.*, adding the agent or modulator to cells in culture) or alternatively, administering the agent or modulator to a subject or patient such that the agent or modulator is capable or contacting the cells of the subject or patient *in vivo*. "Administering" an agent or modulator includes any routine means known in the art or described herein of providing a subject or patient with an agent or modulator. "Coadministering" agents includes administering a first and second agent or modulator, for example, sequentially or coincidentally. In addition to administering agents and/or modulators (*e.g.*, immunomodulatory molecules), certain aspects of the present invention feature administering cells to a subject or patient. For example, cells of a patient (*e.g.*, immune cells or immune effector cells) can be isolated from a subject, contacted with an agent or modulator *in vitro* (*e.g.*, culturing the cells in the presence of the agent or modulator), and administered or readministered to the subject or patient. Routine means can be utilized for isolating immune cells, for example, isolating and/or separating plasma from a subject or patient, isolating bone marrow from a patient or subject, as well as for administering or readministering cells, for example, plasmaphoresis or bone marrow transplants.

The term "subject" includes a living animal, preferably a human subject.

The term "patient" includes a subject, preferably a human subject, in need of treatment (*e.g.*, treatment according to the methodologies of the present invention), potentially in need of treatment, presently undergoing treatment, having or suffering from a disease or disorder which would benefit from at least one methodology of the present invention. Preferably a "patient" is a human patient.

Exemplary diseases and/or disorders from which a patient, as defined herein, may be at risk for, have or be suffering from include but are not limited to burn-associated sepsis, infectious diseases or disorders (*e.g.*, bacterial infection, viral infection, HIV and tuberculosis) cancer, immunodeficiency disorders, AIDS, bone marrow transplant-related immunodeficiency, chemotherapy-related immunodeficiency, allergy, bacterial arthritis, granulomatous disorder, herpes simplex keratitis, autoimmune disease, and various forms of glomerulonephritis including, but not limited to, rhematoid arthritis and multiple sclerosis. Diseases and disorders are to be given their accepted, art-recognized definitions, for example, as set forth in The Physicians Desk Reference.

The phrase "monitoring and immune response", for example, "monitoring a type-1 immune response" includes monitoring the ability of an agent or modulator of the invention to enhance, potentiate, stimulate, upregulate or downregulate or inhibit an immune response, for example, a type-1 or type-2 immune response. In one embodiment, monitoring a type-1 or type-2 response includes determining the level of a detectable indicator of the type-1 or type-2 response. Preferred detectable indicators include cytokines associated with a particular response, cytokine profiles associated with a particular response and/or phenotypic responses. Exemplary detectable indicators of a type-1 response include expression, production or secretion to type-1 cytokines, type-1 cytokine profiles, as well as any other type-1 phenotypic response, as described herein. Exemplary detectable indicators of a type-2 response include expression, production or secretion to type-2 cytokines, type-2 cytokine profiles, as well as any other type-2 phenotypic response, as described herein. In another embodiment, monitoring an immune response further comprises comparing the detectable indicator to a control (*e.g.*, a control profile or control phenotype, for example, the profile or phenotype of the subject or patient prior to treatment or at a previous stage of treatment with an agent or modulator, the profile or phenotype) of a

normal or average subject, or an expected or target profile or phenotype (*e.g.*, a theoretical, desired or predicted profile or phenotype).

Various aspects of the invention are described in further detail in the following subsections.

I. Immunomodulatory Agents

In the immunomodulatory methods of the invention, for example, type-1 immunomodulatory methods, an Eta-1/osteopontin modulator is administered to a subject (*e.g.*, a human subject) or a cell (*e.g.*, a human immune cell) is contacted with the modulator such that an immune response, for example, a type-1 immune response is modulated. In one embodiment, the Eta-1/osteopontin modulator is a "stimulatory agent" (*e.g.*, an agent or modulator that stimulates Eta-1/osteopontin activity), which enhances, potentiated, increases or upregulates a type-1 immune response in a cell or subject. Preferred "stimulatory agents" or "stimulatory modulators" include isolated Eta-1/osteopontin proteins or polypeptides and biologically active fragments thereof, isolated nucleic acid molecules encoding such Eta-1/osteopontin proteins or polypeptides and biologically active fragments thereof, biosynthetic immunomodulatory molecules, Eta-1 peptides, peptidomimetics and small molecule agonists (*e.g.*, Eta-1 peptides, peptidomimetics and small molecule agonists capable of specifically binding to an Eta-1/osteopontin receptor, for example, integrin or CD44, and/or upregulating the activity of the Eta-1/osteopontin receptor as described in further detail below. In another embodiment, the Eta-1/osteopontin modulator is an "inhibitory agent" (*e.g.*, an agent or modulator that inhibits Eta-1/osteopontin activity), which decreases or downregulates a type-1 immune response in a cell or subject. Preferred "inhibitory agents" or "inhibitory modulators" include antisense Eta-1/osteopontin nucleic acid molecules, Eta-1/osteopontin antibodies and/or Eta-1/osteopontin receptor antibodies (*e.g.* compounds capable of specifically binding to an Eta-1/osteopontin receptor, for example, integrin or CD44, and/or downregulating the activity of the Eta-1/osteopontin receptor), as described in further detail below. Additional preferred modulatory agents modulate selected activities of Eta-1/osteopontin, for example, modulate activities resulting from ligation of CD44 and/or integrin by Eta-1/osteopontin. Particularly

preferred modulatory agents modulate immune responses specific for Eta-1/osteopontin interacting with CD44 and/or integrin.

A. Isolated Eta-1/Osteopontin Proteins, Biologically-active Fragments Thereof and

One aspect of the invention pertains to isolated Eta-1/osteopontin proteins and biologically active portions thereof. Eta-1/osteopontin proteins can be isolated from cells or tissue sources by an appropriate purification scheme using standard protein purification techniques, can produced by recombinant DNA techniques or can be synthesized chemically using standard peptide synthesis techniques. Biologically active portions of Eta-1/osteopontin polypeptides can be further generated by enzymatic digestion of full-length Eta-1/osteopontin polypeptides, can be produced by recombinant DNA techniques or can be synthesized chemically using standard peptide synthesis techniques.

An "isolated" or "purified" Eta-1/osteopontin protein or biologically active portion thereof is substantially free of cellular material or other contaminating proteins from the cell or tissue source from which the Eta-1/osteopontin is derived, or substantially free from chemical precursors or other chemicals when chemically synthesized. The language "substantially free of cellular material" includes preparations of Eta-1/osteopontin in which the protein is separated from cellular components of the cells from which it is isolated or recombinantly produced. The language "substantially free of cellular material" includes preparations in which the recombinant molecule is separated from cellular components of the cells from which it is isolated or recombinantly produced. In one embodiment, the language "substantially free of cellular material" includes preparations having less than about 30% (by dry weight) of contaminating cellular material, more preferably less than about 20% of contaminating material, still more preferably less than about 10% of contaminating material, and most preferably less than about 5% contaminating material. When Eta-1/osteopontin is recombinantly produced, it is also preferably substantially free of culture medium, *i.e.*, culture medium represents less than about 20%, more preferably less than about 10%, and most preferably less than about 5% of the volume of the preparation.

The language "substantially free of chemical precursors or other chemicals" includes preparations in which the chemically synthesized molecule is separated from chemical precursors or other chemicals which are involved in the synthesis of the molecule. In one embodiment, the language "substantially free of chemical precursors or other chemicals" includes preparations having less than about 30% (by dry weight) of chemical precursors or contaminating chemicals, more preferably less than about 20% chemical precursors or contaminating chemicals, still more preferably less than about 10% chemical precursors or contaminating chemicals, and most preferably less than about 5% chemical precursors or contaminating chemicals.

In a preferred embodiment, an Eta-1/osteopontin protein for use in the present invention is a human Eta-1/osteopontin. For example, any of the Eta-1/osteopontin proteins set forth as SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6 are suitable for use in the immunomodulatory methods of the present invention (*e.g.*, Eta-1/osteopontin protein-a, Eta-1/osteopontin protein-b or Eta-1/osteopontin protein-c, respectively). Also suitable for use in the immunomodulatory methods of the present invention are Eta-1/osteopontin homologues or variants which vary at the amino acid sequence level when compared, for example, to the Eta-1/osteopontin proteins set forth as SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6 but which retain the biological activity of the Eta-1/osteopontin proteins set forth as SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6. For example, Eta-1/osteopontin homologues or variants having 85-90%, 90-95%, 96%, 97%, 98%, 99% or more sequence identity to the Eta-1/osteopontin proteins set forth as SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6 but which retain biological activity are suitable for use in the immunomodulatory methods of the present invention. Eta-1/osteopontin homologues or variants can have amino acid substitutions (particularly conservative amino acid substitutions) at "non-essential" amino acid residues in the sequence of SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6. A "non-essential" amino acid residue is a residue that can be altered from the sequence set forth in SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6 without altering the biological activity, whereas an "essential" amino acid residue is required for biological activity. For example, amino acid residues that are conserved among proteins or domains of proteins from different species are predicted to be particularly unamenable to alteration.

Alternatively, Eta-1/osteopontin homologues or variants can have a conservative amino acid substitutions at one or more predicted essential or non-essential amino acid residues. A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (*e.g.*, threonine, valine, isoleucine) and aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan, histidine). Thus, a predicted essential or nonessential amino acid residue is preferably replaced with another amino acid residue from the same side chain family.

Also suitable for use in the immunomodulatory methods of the present invention are Eta-1/osteopontin homologues or variants which are encoded by nucleic acid molecules comprising the nucleotide sequences of SEQ ID NO:1, SEQ ID NO:3 or SEQ ID NO:5, as well as Eta-1/osteopontin homologues or variants encoded by nucleic acid molecules having 85-90%, 90-95%, 96%, 97%, 98%, 99% or more sequence identity to the Eta-1/osteopontin nucleotide sequences of SEQ ID NO:1, SEQ ID NO:3 or SEQ ID NO:5 and/or isolated nucleic acid molecules which hybridize under stringent hybridization conditions to the Eta-1/osteopontin nucleotide sequences of SEQ ID NO:1, SEQ ID NO:3 or SEQ ID NO:5. As used herein, the term "hybridizes under stringent conditions" is intended to describe conditions for hybridization and washing under which nucleotide sequences at least 90% homologous to each other typically remain hybridized to each other. Such stringent conditions are known to those skilled in the art and can be found in *Current Protocols in Molecular Biology*, John Wiley & Sons, N.Y. (1992). A preferred, non-limiting example of stringent hybridization conditions are hybridization in 6X sodium chloride/sodium citrate (SSC) at about 45°C, followed by one or more washes in 0.2 X SSC, 0.1% SDS at 50-65°C.

Biologically active portions of Eta-1/osteopontin include fragments or portions sufficiently homologous to Eta-1/osteopontin, *e.g.*, the amino acid sequence shown in SEQ ID NO:2, which include less amino acids than the full length polypeptide,

and exhibit at least one activity of full-length Eta-1/osteopontin. Typically, biologically active portions comprise a domain with at least one activity of the full-length polypeptide. A biologically active portion can be a polypeptide which is, for example, 50-100, 100-150, 150-200, 200-250, 250-300 or more amino acids in length.

In one embodiment, a biologically active portion of Eta-1/osteopontin comprises an IL-12 stimulatory domain of Eta-1/osteopontin. As used herein, an IL-12 stimulatory domain is a domain of Eta-1/osteopontin capable of ligating integrin (*e.g.*, αvβ3 integrin) such that IL-12 production is stimulated by the cell. In a preferred embodiment, an IL-12 stimulatory domain is incapable of ligating CD44 expressed on the cell. In one embodiment, an IL-12 stimulatory domain is about 50-60, 60-70, 70-80, 80-90 or 90-100 amino acid residues in length. In another embodiment, an IL-12 stimulatory domain is of a size sufficient to induce IL-12 production by a cell but includes insufficient amino acid residues to inhibit IL-10 production by the cell. A particularly preferred IL-12 stimulatory domain includes the residues of the fragment NK10 described herein.

In another embodiment, a biologically active portion of Eta-1/osteopontin comprises an IL-10 inhibitory domain of Eta-1/osteopontin. As used herein, an IL-10 inhibitory domain is a domain of Eta-1/osteopontin capable of ligating CD44 such that IL-10 production is inhibited by the cell. In a preferred embodiment, an IL-10 inhibitory domain is incapable of ligating integrin (*e.g.*, αvβ3 integrin) expressed on the cell. In one embodiment, an IL-10 inhibitory domain is about 50-60, 60-70, 70-80, 80-90 or 90-100 amino acid residues in length. In another embodiment, an IL-10 inhibitory domain is of a size sufficient to inhibit IL-10 production by a cell but includes insufficient amino acid residues to stimulate IL-12 production by the cell. A particularly preferred IL-10 inhibitory domain includes about amino acid residues 169-200, 169-220, 169-240, 169-260, or 169-280 of SEQ ID NO:2.

To determine the percent homology of two amino acid sequences or of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (*e.g.*, gaps can be introduced in the sequence of a first amino acid or nucleic acid sequence for optimal alignment with a second amino or nucleic acid sequence). The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the

same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are homologous at that position (*i.e.*, as used herein amino acid or nucleic acid "homology" is equivalent to amino acid or nucleic acid "identity"). The percent homology between the two sequences is a function of the number of identical positions shared by the sequences (*i.e.*, % homology = # of identical positions/total # of positions x 100). The determination of percent homology between two sequences can be accomplished using a mathematical algorithm. A preferred, non-limiting example of a mathematical algorithm utilized for the comparison of two sequences is the algorithm of Karlin and Altschul (1990) *Proc. Natl. Acad. Sci. USA* 87:2264-68, modified as in Karlin and Altschul (1993) *Proc. Natl. Acad. Sci. USA* 90:5873-77. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul *et al.* (1990) *J. Mol. Biol.* 215:403-10. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to the nucleic acid molecule sequences of the invention. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to the protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul *et al.* (1997) *Nucleic Acids Research* 25(17):3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) can be used. See http://www.ncbi.nlm.nih.gov. Another preferred, non-limiting example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Myers and Miller (1988) *Comput Appl Biosci.* 4:11-17. Such an algorithm is incorporated into the ALIGN program available, for example, at the GENESTREAM network server, IGH Montpellier, FRANCE (http://vega.igh.cnrs.fr) or at the ISREC server (http://www.ch.embnet.org). When utilizing the ALIGN program for comparing amino acid sequences, a PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used.

The invention also provides chimeric or fusion proteins, for example, recombinant chimeric or fusion proteins designed to facilitate the purification of Eta-1/osteopontin (*e.g.*, GST-fusion proteins or HA-tagged fusion proteins). Also provided are chimeric or fusion proteins (*e.g.*, Eta-1/osteopontin containing a heterologous signal

sequence at its N-terminus) to enhance expression and/or secretion recombinant Eta-1/osteopontin. Chimeric or fusion proteins of the invention are produced by standard recombinant DNA techniques as described, for example, in *Current Protocols in Molecular Biology*, eds. Ausubel et al. John Wiley & Sons: 1992). Moreover, many expression vectors are commercially available that already encode a fusion moiety (*e.g.*, a GST polypeptide). An Eta-1/osteopontin-encoding nucleic acid, as described herein, can be cloned into such an expression vector such that the fusion moiety is linked in-frame to the Eta-1/osteopontin protein.

Also featured are Eta-1/osteopontin proteins and biologically active portions which are incorporated into pharmaceutical compositions as described herein.

B. Isolated Nucleic Acid Molecules, Vectors, Host Cells

Another aspect of the invention pertains to isolated nucleic acid molecules that encode Eta-1/osteopontin proteins or portions or biologically active fragments thereof. The term "nucleic acid molecule" includes DNA molecules (e.g., cDNA or genomic DNA) and RNA molecules (e.g., mRNA) and analogs of the DNA or RNA generated using nucleotide analogs. The nucleic acid molecule can be single-stranded or double-stranded, but preferably is double-stranded DNA.

An "isolated" nucleic acid molecule is one which is separated from other nucleic acid molecules which are present in the natural source of the nucleic acid. Preferably, an "isolated" nucleic acid is free of sequences which naturally flank the nucleic acid (i.e., sequences located at the 5' and 3' ends of the nucleic acid) in the genomic DNA of the organism from which the nucleic acid is derived. For example, in various embodiments, the isolated nucleic acid molecule can contain less than about 5 kb, 4kb, 3kb, 2kb, 1 kb, 0.5 kb or 0.1 kb of nucleotide sequences which naturally flank the nucleic acid molecule in genomic DNA of the cell from which the nucleic acid is derived. Moreover, an "isolated" nucleic acid molecule, such as a cDNA molecule, can be substantially free of other cellular material, or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized.

In another preferred embodiment, an isolated nucleic acid molecule of the invention comprises a nucleotide sequence shown in SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5 or a portion thereof. Also included within the scope of the invention are isolated nucleic acid molecules which are complementary to the nucleotide sequences shown in SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5 or a portion thereof. Also included within the scope of the present invention are isolated nucleic acid molecules which hybridize (*e.g.*, under stringent hybridization conditions) to a complement of the nucleotide sequences shown in SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5 or a portion thereof, thereby forming a stable duplex. Also included within the scope of the present invention are isolated nucleic acid molecules having 80-85%, 90-95%, 96%, 97%, 98%, 99% or more homology or identity to the nucleotide sequences shown in SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5 or a portion thereof. Also included within the scope of the present invention are isolated nucleic acid molecules which are antisense to the nucleic acid molecules shown in SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5. The invention further encompasses nucleic acid molecules that differ from the nucleotide sequence shown in SEQ ID NO:1, SEQ ID NO:3 or SEQ ID NO:5 yet due to degeneracy of the genetic code encode the same molecules as those encoded by the nucleotide sequence shown in SEQ ID NO:1, SEQ ID NO:3 or SEQ ID NO:5.

A nucleic acid molecule of the invention, or portion thereof, can be amplified using cDNA, mRNA or alternatively, genomic DNA, as a template and appropriate oligonucleotide primers according to standard PCR amplification techniques. The nucleic acid so amplified can be cloned into an appropriate vector and characterized by DNA sequence analysis. Furthermore, oligonucleotides (*e.g.*, probes and/or primers) and antisense nucleic acid molecules can be prepared by standard synthetic techniques, *e.g.*, using an automated DNA synthesizer. Oligonucleotides for use in the present invention typically comprises substantially purified oligonucleotide. The oligonucleotide typically comprises a region of nucleotide sequence that hybridizes under stringent conditions to at least about 12, preferably about 25, more preferably about 40, 50 or 75 consecutive nucleotides of the nucleotide sequence of SEQ ID NO:1, SEQ ID NO:3 or SEQ ID NO:5 of or of the complement of the nucleotide sequence of SEQ ID NO:1, SEQ ID NO:3 or SEQ ID NO:5. Also included within the scope of the present invention are oligonucleotides at least 15, 30, 50, 100, 250 or 500 nucleotides in

length which hybridizes under stringent conditions to the nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO:1, SEQ ID NO:3 or SEQ ID NO:5.

A nucleic acid fragment encoding a "biologically active" portion of an Eta-1/osteopontin molecule of the present invention can be prepared by isolating a portion of SEQ ID NO:1, SEQ ID NO:3 or SEQ ID NO:5 which encodes a polypeptide having a biological activity of the naturally-occurring protein from which the portion was derived, expressing the encoded portion of the naturally-occurring protein (*e.g.*, by recombinant expression *in vitro*) and assessing the activity of the encoded portion of the naturally-occurring protein. As used herein, a "naturally-occurring" nucleic acid molecule or protein molecule refers to a molecule having a nucleotide or amino acid sequence that occurs in nature (*e.g.*, a nucleic acid molecule that encodes a natural protein).

Another aspect of the invention pertains to vectors, preferably expression vectors, containing a nucleic acid molecule of the present invention. As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional DNA segments can be ligated. Another type of vector is a viral vector, wherein additional DNA segments can be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (*e.g.*, bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (*e.g.*, non-episomal mammalian vectors) are integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "expression vectors". In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" can be used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors, such as viral vectors (*e.g.*, replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions.

The recombinant expression vectors of the invention comprise a nucleic acid of the invention in a form suitable for expression of the nucleic acid in a host cell, which means that the recombinant expression vectors include one or more regulatory sequences, selected on the basis of the host cells to be used for expression, which is operatively linked to the nucleic acid sequence to be expressed. Within a recombinant expression vector, "operably linked" means that the nucleotide sequence of interest is linked to the regulatory sequence(s) in a manner which allows for expression of the nucleotide sequence (e.g., in an *in vitro* transcription/translation system or in a host cell when the vector is introduced into the host cell). The term "regulatory sequence" includes promoters, enhancers and other expression control elements (e.g., polyadenylation signals). Such regulatory sequences are described, for example, in Goeddel; *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990). Regulatory sequences include those which direct constitutive expression of a nucleotide sequence in many types of host cell and those which direct expression of the nucleotide sequence only in certain host cells (e.g., tissue-specific regulatory sequences). It will be appreciated by those skilled in the art that the design of the expression vector can depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, etc. The expression vectors of the invention can be introduced into host cells to thereby produce proteins or peptides, including fusion proteins or peptides, encoded by nucleic acids as described herein.

The recombinant expression vectors of the invention can be designed for expression in prokaryotic or eukaryotic cells. For example, recombinant proteins can be expressed in bacterial cells such as *E. coli*, insect cells (using baculovirus expression vectors) yeast cells or mammalian cells. Suitable host cells are discussed further in Goeddel, *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990). Alternatively, the recombinant expression vector can be transcribed and translated *in vitro*, for example using T7 promoter regulatory sequences and T7 polymerase.

Expression of proteins in prokaryotes is most often carried out in *E. coli* with vectors containing constitutive or inducible promotors directing the expression of either fusion or non-fusion proteins. Fusion vectors add a number of amino acids to a protein encoded therein, usually to the amino terminus of the recombinant protein. Such

fusion vectors typically serve three purposes: 1) to increase expression of recombinant protein; 2) to increase the solubility of the recombinant protein; and 3) to aid in the purification of the recombinant protein by acting as a ligand in affinity purification. Often, in fusion expression vectors, a proteolytic cleavage site is introduced at the junction of the fusion moiety and the recombinant protein to enable separation of the recombinant protein from the fusion moiety subsequent to purification of the fusion protein. Such enzymes, and their cognate recognition sequences, include Factor Xa, thrombin and enterokinase. Typical fusion expression vectors include pGEX (Pharmacia Biotech Inc; Smith, D.B. and Johnson, K.S. (1988) *Gene* 67:31-40), pMAL (New England Biolabs, Beverly, MA) and pRIT5 (Pharmacia, Piscataway, NJ) which fuse glutathione S-transferase (GST), maltose E binding protein, or protein A, respectively, to the target recombinant protein.

Examples of suitable inducible non-fusion *E. coli* expression vectors include pTrc (Amann et al., (1988) *Gene* 69:301-315) and pET 11d (Studier et al., *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, California (1990) 60-89). Target gene expression from the pTrc vector relies on host RNA polymerase transcription from a hybrid trp-lac fusion promoter. Target gene expression from the pET 11d vector relies on transcription from a T7 gn10-lac fusion promoter mediated by a coexpressed viral RNA polymerase (T7 gn1). This viral polymerase is supplied by host strains BL21(DE3) or HMS174(DE3) from a resident λ prophage harboring a T7 gn1 gene under the transcriptional control of the lacUV 5 promoter.

One strategy to maximize recombinant protein expression in *E. coli* is to express the protein in a host bacteria with an impaired capacity to proteolytically cleave the recombinant protein (Gottesman, S., *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, California (1990) 119-128). Another strategy is to alter the nucleic acid sequence of the nucleic acid to be inserted into an expression vector so that the individual codons for each amino acid are those preferentially utilized in *E. coli* (Wada et al., (1992) *Nucleic Acids Res.* 20:2111-2118). Such alteration of nucleic acid sequences of the invention can be carried out by standard DNA synthesis techniques.

In another embodiment, the expression vector is a yeast expression vector. Examples of vectors for expression in yeast *S. cerivisae* include pYepSec1 (Baldari, et al., (1987) *Embo J.* 6:229-234), pMFa (Kurjan and Herskowitz, (1982) *Cell* 30:933-943), pJRY88 (Schultz et al., (1987) *Gene* 54:113-123), pYES2 (Invitrogen Corporation, San Diego, CA), and picZ (InVitrogen Corp, San Diego, CA).

Alternatively, recombinant proteins can be expressed in insect cells using baculovirus expression vectors. Baculovirus vectors available for expression of proteins in cultured insect cells (e.g., Sf 9 cells) include the pAc series (Smith et al. (1983) *Mol. Cell Biol.* 3:2156-2165) and the pVL series (Lucklow and Summers (1989) *Virology* 170:31-39).

In yet another embodiment, a nucleic acid of the invention is expressed in mammalian cells using a mammalian expression vector. Examples of mammalian expression vectors include pCDM8 (Seed, B. (1987) *Nature* 329:840) and pMT2PC (Kaufman et al. (1987) *EMBO J.* 6:187-195). When used in mammalian cells, the expression vector's control functions are often provided by viral regulatory elements. For example, commonly used promoters are derived from polyoma, Adenovirus 2, cytomegalovirus and Simian Virus 40. For other suitable expression systems for both prokaryotic and eukaryotic cells see chapters 16 and 17 of Sambrook, J., Fritsh, E. F., and Maniatis, T. *Molecular Cloning: A Laboratory Manual. 2nd, ed., Cold Spring Harbor Laboratory,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.

In another embodiment, the recombinant mammalian expression vector is capable of directing expression of the nucleic acid preferentially in a particular cell type (*e.g.*, tissue-specific regulatory elements are used to express the nucleic acid). Tissue-specific regulatory elements are known in the art. Non-limiting examples of suitable tissue-specific promoters include the albumin promoter (liver-specific; Pinkert *et al.* (1987) *Genes Dev.* 1:268-277), lymphoid-specific promoters (Calame and Eaton (1988) *Adv. Immunol.* 43:235-275), in particular promoters of T cell receptors (Winoto and Baltimore (1989) *EMBO J.* 8:729-733) and immunoglobulins (Banerji *et al.* (1983) *Cell* 33:729-740; Queen and Baltimore (1983) *Cell* 33:741-748), neuron-specific promoters (e.g., the neurofilament promoter: Byrne and Ruddle (1989) *PNAS* 86:5473-5477), pancreas-specific promoters (Edlund *et al.* (1985) *Science* 230:912-916), and mammary

gland-specific promoters (*e.g.*, milk whey promoter; U.S. Patent No. 4,873,316 and European Application Publication No. 264,166). Developmentally-regulated promoters are also encompassed, for example the murine hox promoters (Kessel and Gruss (1990) *Science* 249:374-379) and the α-fetoprotein promoter (Campes and Tilghman (1989) *Genes Dev.* 3:537-546).

The invention further provides a recombinant expression vector comprising a DNA molecule of the invention cloned into the expression vector in an antisense orientation. That is, the DNA molecule is operatively linked to a regulatory sequence in a manner which allows for expression (by transcription of the DNA molecule) of an RNA molecule which is antisense to an mRNA corresponding to a nucleic acid molecule of the present invention. Regulatory sequences operatively linked to a nucleic acid cloned in the antisense orientation can be chosen which direct the continuous expression of the antisense RNA molecule in a variety of cell types, for instance viral promoters and/or enhancers, or regulatory sequences can be chosen which direct constitutive, tissue specific or cell type specific expression of antisense RNA. The antisense expression vector can be in the form of a recombinant plasmid, phagemid or attenuated virus in which antisense nucleic acids are produced under the control of a high efficiency regulatory region, the activity of which can be determined by the cell type into which the vector is introduced. For a discussion of the regulation of gene expression using antisense genes see Weintraub, H. et al., Antisense RNA as a molecular tool for genetic analysis, *Reviews - Trends in Genetics*, Vol. 1(1) 1986.

Another aspect of the invention pertains to host cells into which a recombinant expression vector of the invention has been introduced. The terms "host cell" and "recombinant host cell" are used interchangeably herein. It is understood that such terms refer not only to the particular subject cell but to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

A host cell can be any prokaryotic or eukaryotic cell. For example, Immunomodulin protein can be expressed in bacterial cells such as *E. coli*, insect cells, yeast or mammalian cells (such as Chinese hamster ovary cells (CHO) or COS cells). Other suitable host cells are known to those skilled in the art.

Vector DNA can be introduced into prokaryotic or eukaryotic cells via conventional transformation or transfection techniques. As used herein, the terms "transformation" and "transfection" are intended to refer to a variety of art-recognized techniques for introducing foreign nucleic acid (e.g., DNA) into a host cell, including calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, or electroporation. Suitable methods for transforming or transfecting host cells can be found in Sambrook, et al. (*Molecular Cloning: A Laboratory Manual. 2nd, ed., Cold Spring Harbor Laboratory,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989), and other laboratory manuals.

For stable transfection of mammalian cells, it is known that, depending upon the expression vector and transfection technique used, only a small fraction of cells may integrate the foreign DNA into their genome. In order to identify and select these integrants, a gene that encodes a selectable marker (*e.g.*, resistance to antibiotics) is generally introduced into the host cells along with the gene of interest. Preferred selectable markers include those which confer resistance to drugs, such as G418, hygromycin and methotrexate. Nucleic acid encoding a selectable marker can be introduced into a host cell on the same vector as that encoding recombinant proteins or can be introduced on a separate vector. Cells stably transfected with the introduced nucleic acid can be identified by drug selection (*e.g.*, cells that have incorporated the selectable marker gene will survive, while the other cells die).

A host cell of the invention, such as a prokaryotic or eukaryotic host cell in culture, can be used to produce (*i.e.*, express) recombinant protein. Accordingly, the invention further provides methods for producing recombinant protein using the host cells of the invention. In one embodiment, the method comprises culturing the host cell of invention (into which a recombinant expression vector encoding recombinant protein has been introduced) in a suitable medium such that the recombinant protein is produced. In another embodiment, the method further comprises isolating the recombinant protein from the medium or the host cell.

C. Activating, Neutralizing and/or Blocking Antibodies

As described herein, preferred Eta-1 modulators are agents that are capable of modulating select Eta-1/osteopontin-mediated activities, in particular, select activities associated with potentiation of a type-1 immune response. Accordingly, in one embodiment, the invention features methods of modulating an immune response which include administering an Eta-1 antibody, for example, an antibody which specifically block or neutralizes the interaction of Eta-1/osteopontin with a cell surface receptor (*e.g.*, CD44 and/or integrin $\alpha v\beta 3$). In one embodiment, an antibody is specific for the N-terminal, *e.g.*, IL-12 stimulatory domain of Eta-1/osteopontin, as defined herein. In another embodiment, the antibody is specific for the C-terminal, *e.g.*, IL-10 inhibitory domain if Eta-1/osteopontin. In yet another embodiment, the antibody is specific for the RGD sequence of Eta-1/osteopontin (*e.g.*, the integrin binding domain). In a preferred embodiment, the antibody is LF123, as described herein. Also included within the scope of the present invention are fragments of such antibodies, *e.g.*, Fab' fragments, humanized antibodies, and the like, for use as therapeutic agents.

D. Peptides, Peptidomimetics and Small Molecule Modulators

The present invention also pertains to Eta-1/osteopontin peptides, Eta-1/osteopontin peptidomimetics and or small molecule modulators of Eta-1/osteopontin which function as either Eta-1/osteopontin agonists (mimetics) or as Eta-1/osteopontin antagonists. An Eta-1/osteopontin agonists can retain substantially the same, or a subset, of the biological activities of the naturally occurring form of Eta-1/osteopontin. An Eta-1/osteopontin antagonist can inhibit one or more of the activities of the naturally occurring form of Eta-1/osteopontin. Thus, specific biological effects can be elicited by treatment with an Eta-1/osteopontin agonist or antagonist of limited function. In one embodiment, treatment of a subject with an Eta-1/osteopontin agonist or antagonist having a subset of the biological activities of the naturally occurring form of the protein has fewer side effects in a subject relative to treatment with the full length Eta-1/osteopontin.

Eta-1/osteopontin agonist or antagonist can be identified by screening libraries of Eta-1 peptides, combinatorial libraries based on Eta-1 peptides or small molecule libraries for Eta-1 agonist or antagonist activity. In one embodiment, a variegated library is generated by combinatorial mutagenesis at the Eta-1/osteopontin nucleic acid level and is encoded by a variegated gene library. Variegated libraries of compounds can be produced by, for example, enzymatically ligating a mixture of synthetic oligonucleotides into gene sequences such that a degenerate set of potential Eta-1/osteopontin sequences is expressible as individual polypeptides, or alternatively, as a set of larger fusion proteins (e.g., for phage display) containing the set of Eta-1/osteopontin sequences therein. There are a variety of methods which can be used to produce libraries of potential Eta-1/osteopontin variants from a degenerate oligonucleotide sequence. Chemical synthesis of a degenerate gene sequence can be performed in an automatic DNA synthesizer, and the synthetic gene then ligated into an appropriate expression vector. Use of a degenerate set of genes allows for the provision, in one mixture, of all of the sequences encoding the desired set of potential Eta-1/osteopontin sequences. Methods for synthesizing degenerate oligonucleotides are known in the art (see, e.g., Narang, S.A. (1983) *Tetrahedron* 39:3; Itakura et al. (1984) *Annu. Rev. Biochem.* 53:323; Itakura et al. (1984) *Science* 198:1056; Ike et al. (1983) *Nucleic Acid Res.* 11:477.

In addition, libraries of fragments of Eta-1/osteopontin coding sequence can be used to generate a variegated population of Eta-1/osteopontin fragments for screening and subsequent selection of variants of Eta-1/osteopontin. In one embodiment, a library of coding sequence fragments can be generated by treating a double stranded PCR fragment of an Eta-1/osteopontin coding sequence with a nuclease under conditions wherein nicking occurs only about once per molecule, denaturing the double stranded DNA, renaturing the DNA to form double stranded DNA which can include sense/antisense pairs from different nicked products, removing single stranded portions from reformed duplexes by treatment with S1 nuclease, and ligating the resulting fragment library into an expression vector. By this method, an expression library can be derived which encodes N-terminal, C-terminal and internal fragments of various sizes of the Eta-1/osteopontin protein.

Additional exemplary methods for the synthesis of molecular libraries can be found in the art, for example in: DeWitt *et al.* (1993) *Proc. Natl. Acad. Sci. U.S.A.* 90:6909; Erb *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:11422; Zuckermann *et al.* (1994). *J. Med. Chem.* 37:2678; Cho et al. (1993) *Science* 261:1303; Carrell *et al.* (1994) *Angew. Chem. Int. Ed. Engl.* 33:2059; Carell *et al.* (1994) *Angew. Chem. Int. Ed. Engl.* 33:2061; and in Gallop *et al.* (1994) *J. Med. Chem.* 37:1233.

Libraries of compounds may be presented in solution (e.g., Houghten (1992) *Biotechniques* 13:412-421), or on beads (Lam (1991) *Nature* 354:82-84), chips (Fodor (1993) *Nature* 364:555-556), bacteria (Ladner USP 5,223,409), spores (Ladner USP '409), plasmids (Cull *et al.* (1992) *Proc Natl Acad Sci USA* 89:1865-1869) or on phage (Scott and Smith (1990) *Science* 249:386-390); (Devlin (1990) *Science* 249:404-406); (Cwirla *et al.* (1990) *Proc. Natl. Acad. Sci.* 87:6378-6382); (Felici (1991) *J. Mol. Biol.* 222:301-310); (Ladner *supra.*).

Additional test compounds can be obtained using any of the numerous approaches in combinatorial library methods known in the art, including: biological libraries; spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the 'one-bead one-compound' library method; and synthetic library methods using affinity chromatography selection. The biological library approach is limited to peptide libraries, while the other four approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds (Lam, K.S. (1997) *Anticancer Drug Des.* 12:145).

A. Screening Assays:

Several techniques are known in the art for screening libraries (*e.g.*, combinatorial libraries or small molecule libraries) for compounds having a selected property. Such techniques are preferably adaptable for rapid screening of the libraries described herein. Particularly preferred techniques are those which are amenable to high through-put analysis.

For example, candidate or test compounds can be screened for their ability to modulate the interaction of Eta-1/osteopontin with a CD44 or integrin receptor. In one embodiment, an assay is a cell-based assay in which a cell which expresses CD44 or integrin on the cell surface is contacted with a test compound.

optionally in the presence of Eta-1/osteopontin, and the ability of the test compound to modulate the interaction of Eta-1/osteopontin with a CD44 or integrin receptor is determined. The cell can be of mammalian origin, for example, a macrophage. Determining the ability of the test compound to modulate the interaction of Eta-1/osteopontin with a CD44 or integrin receptor can be accomplished, for example, by coupling the test compound (or Eta-1/osteopontin) with a radioisotope or enzymatic label such that binding to CD44 or integrin can be determined by detecting the labeled compound in a complex. For example, test compounds can be labeled with ^{125}I, ^{35}S, ^{14}C, or ^{3}H, either directly or indirectly, and the radioisotope detected by direct counting of radioemmission or by scintillation counting. Alternatively, reagents can be enzymatically labeled with, for example, horseradish peroxidase, alkaline phosphatase, or luciferase, and the enzymatic label detected by determination of conversion of an appropriate substrate to product.

The ability of a test compound to modulate the interaction of Eta-1/osteopontin with a CD44 or integrin receptor can also be determined without the labeling of any of the interactants. For example, a microphysiometer can be used to detect the interaction of components without the labeling of either the test compound or the receptor. McConnell, H. M. *et al.* (1992) *Science* 257:1906-1912. Determining the ability to modulate the interaction of Eta-1/osteopontin with a CD44 or integrin receptor can also be accomplished by determining, for example, induction of a cellular second messenger (i.e. intracellular Ca^{2+}, diacylglycerol, IP_3, etc.), detecting the induction of a reporter gene, or detecting a cellular response, for example, a proliferative response or an inflammatory response.

In yet another embodiment, an assay of the present invention is a cell-free assay in which an Eta-1/osteopontin protein or biologically active portion thereof is contacted with a test compound and the ability of the test compound to bind to the Eta-1/osteopontin protein or biologically active portion thereof is determined. Binding of the test compound to the Eta-1/osteopontin protein can be determined either directly or indirectly as described above. Binding of the test compound to the Eta-1/osteopontin protein can also be accomplished using a technology such as real-time Biomolecular Interaction Analysis (BIA). Sjolander, S. and Urbaniczky, C. (1991) *Anal. Chem.* 63:2338-2345 and Szabo *et al.* (1995) *Curr. Opin. Struct. Biol.* 5:699-705. As used

herein, "BIA" is a technology for studying biospecific interactions in real time, without labeling any of the interactants (e.g., BIAcore™). Changes in the optical phenomenon of surface plasmon resonance (SPR) can be used as an indication of real-time reactions between biological molecules.

In a preferred embodiment, the assay includes contacting the Eta-1/osteopontin protein or biologically active portion thereof with a known ligand which binds Eta-1/osteopontin to form an assay mixture, contacting the assay mixture with a test compound, and determining the ability of the test compound to interact with an Eta-1/osteopontin protein, wherein determining the ability of the test compound to interact with an Eta-1/osteopontin protein comprises determining the ability of the test compound to preferentially bind to Eta-1/osteopontin or biologically active portion thereof as compared to the known ligand.

In another embodiment, the assay is a cell-free assay in which a CD44 receptor or integrin receptor is contacted with a test compound (and optionally with Eta-1/osteopontin) and the ability of the test compound to modulate (e.g., stimulate or inhibit) the resulting interaction is determined. Cell-free assays of the present invention are amenable to use of both soluble and/or membrane-bound forms of receptors. In the case of cell-free assays in which a receptor is used it may be desirable to utilize a solubilizing agent such that the membrane-bound form of the isolated protein is maintained in solution. Examples of such solubilizing agents include non-ionic detergents such as n-octylglucoside, n-dodecylglucoside, n-dodecylmaltoside, octanoyl-N-methylglucamide, decanoyl-N-methylglucamide, Triton® X-100, Triton® X-114, Thesit®, Isotridecypoly(ethylene glycol ether)$_n$, 3-[(3-cholamidopropyl)dimethylamminio]-1-propane sulfonate (CHAPS), 3-[(3-cholamidopropyl)dimethylamminio]-2-hydroxy-1-propane sulfonate (CHAPSO), or N-dodecyl=N,N-dimethyl-3-ammonio-1-propane sulfonate.

In more than one of the above assay methods, it may be desirable to immobilize at least one assay reagent to facilitate separation of complexed from uncomplexed forms of one or both of the reagents, as well as to accommodate automation of the assay, for example, glutathione-S-transferase/ fusion proteins can be adsorbed onto glutathione sepharose beads (Sigma Chemical, St. Louis, MO) or glutathione derivatized microtitre plates. Alternatively at least one reagent can be immobilized utilizing conjugation of biotin and

streptavidin. Alternatively, antibodies reactive at least one reagent can be derivatized to wells or plates to immobilize reagents.

Novel agents identified by the above-described screening assays can be tested in an appropriate animal model, for example, to determine the efficacy, toxicity, or side effects of treatment with such an agent. Alternatively, agent can be tested in at least one of the *in vitro* or *in situ* assays described herein.

II. Biosynthetic Immunomodulatory Molecules

Based on the discovery of an immunomodulatory function of Eta-1/osteopontin, and in particular, the discovery of the IL-12 stimulatory and IL-10 inhibitory domains of Eta-1/osteopontin, the present invention features biosynthetic molecules which are modeled after these key functional domains. The biosynthetic molecules are useful in regulating a variety of cellular processes as well as in modulating immune responses. In particular, the biosynthetic immunomodulatory molecules are useful in biasing an immune response from a type-1 to a type-2 immune response.

As used herein, the term "biosynthetic molecule" includes molecules which have a biological activity and which are built or synthesized by the combination or union of components or elements that are simpler than the biosynthesized molecule. A biosynthetic molecule of the present invention is made or built by the hand of man (including automated processes) and accordingly, is distinguishable from a naturally-occurring molecule which is results from a naturally-occurring biological process. Alternatively, an organism can be used to produce a biosynthetic molecule of the present invention, provided that at least at one step in the synthesis, there is the intervention of man.

Accordingly, in one embodiment, the present invention features biosynthetic immunomodulatory molecules which include an IL-12 stimulatory component and a biomodular component, forming a molecule which modulates an immune response. The term "IL-12 stimulatory component" includes a piece or constituent of a molecule (*e.g.*, a fragment of Eta-1/osteopontin) which is smaller than the molecule of which it is a part, which functions to stimulate, enhance, upregulate the expression, production and/or secretion of the cytokine, IL-12, from a cell. A molecule which includes an IL-12 stimulatory component, for example, is capable of causing a cell capable of

expressing, producing and/or secreting IL-12..to express and/or secrete more of the cytokine in the presence of the IL-12 stimulatory component than in the absence of the IL-12 stimulatory component.

In another embodiment, the present invention features biosynthetic immunomodulatory molecules which include an IL-10 inhibitory component and a biomodular component, forming a molecule which modulates an immune response. The term "IL-10 inhibitory component" includes a piece or constituent of a molecule which is smaller than the molecule of which it is a part, which functions to inhibit the expression and/or secretion of the cytokine, IL-10, from a cell. A molecule which includes an IL-10 inhibitory component, for example, is capable of causing a cell capable of expressing and/or secreting IL-10, to express and/or secrete less of the cytokine in the presence of the IL-10 inhibitory component than in the absence of the IL-10 inhibitory component.

In addition to the IL-12 stimulatory component or the IL-10 inhibitory component defined herein, the biosynthetic immunomodulatory molecules of the present invention can include a biomodular component. The term "biomodular component" includes a piece or constituent of a molecule which is smaller than the molecule of which it is a part, which has either a biological function which is distinct from that of the IL-12 stimulatory component, the IL-10 inhibitory component or has a biological structure which is distinct from that of the IL-12 stimulatory component or the IL-10 inhibitory component. A biomodular component is a piece or constituent that either is not found in a naturally-occurring molecule which includes an IL-12 stimulatory component or an IL-10 inhibitory component (*e.g.*, Eta-1/osteopontin) or is not found in the same proximal relation to an IL-12 stimulatory component or an IL-10 inhibitory component as it exists within a naturally-occurring molecule. In one embodiment, a biomodular component is a polypeptide. Polypeptide biomodular components of the present invention include, but are not limited to signal peptides, a calcium/apatite binding domains and a heparin binding domains.

The term "signal peptide" or "signal sequence" refers to a peptide containing about 20 amino acids which occurs at the N-terminus of secretory and integral membrane proteins and which contains a large number of hydrophobic amino acid residues. For example, a signal sequence contains at least about 14-28 amino acid

residues, preferably about 16-26 amino acid residues, more preferably about 18-24 amino acid residues, and more preferably about 20-22 amino acid residues, and has at least about 40-70%, preferably about 50-65%, and more preferably about 55-60% hydrophobic amino acid residues (e.g., Alanine, Valine, Leucine, Isoleucine, Phenylalanine, Tyrosine, Tryptophan, or Proline). Such a "signal sequence", also referred to in the art as a "signal peptide", serves to direct a protein containing such a sequence from the endoplasmic reticulum of a cell to the golgi apparatus and ultimately to a lipid bilayer (*e.g.*, for secretion).

The term "calcium/apatite binding domain" includes a domain which, when included within a protein, polypeptide, or biosynthetic molecule of the present invention, functions to bind calcium, bind metal ions, or bind apatite (*e.g.*, hydroxyapatite). A "calcium/apatite binding domain" can also be referred to as a "6-Asp" domain. Also preferred is a 6-His domain. 6-Asp and 6-His domains are particularly useful for purification of the biosynthetic molecules of the preset invention.

The term "heparin binding domain" includes a domain which, when included within a protein, polypeptide, or biosynthetic molecule of the present invention, functions to bind the protein, polypeptide, or biosynthetic molecule to heparin. A "heparin binding domain" further includes a domain which has within it at least one, preferably two, three, four, five, six, or more "heparin binding domain minimum repeating units". The term "heparin binding domain minimum repeating unit" includes the consensus motif basic residue - basic residue - any residue - basic residue. Preferably, a "heparin binding domain minimum repeating unit" has the sequence arginine – arginine – any residue – arginine. Also preferred are collagen binding domains. Heparin binding domains and/or collagen binding domains are particularly useful for stabilizing the biosynthetic molecules of the present invention, *e.g.*, for anchoring or adhering the molecules to ECM surrounding target cells of the invention.

Accordingly, a biosynthetic immunomodulatory molecule of the present invention is formed by the combination of at least an IL-12 stimulatory component or an IL-10 inhibitory domain and a biomodular component. The term "formed" or "forming" includes the bringing together of at least two components into a structural and/or functional association. For example, a recombinant nucleic acid molecule can be formed by the bringing together of at least two nucleic acid components. Alternatively,

a recombinant protein can be formed by the bringing together of at least two protein components. Moreover, a composition can be formed by the bringing together of at least two compositions.

In a preferred embodiment, the present invention features biosynthetic molecules which include an IL-10 inhibitory component which is derived from Eta-1/osteopontin. A component "derived from", for example, Eta-1/osteopontin, includes a component which has certain features which originate from Eta-1/osteopontin and are recognizable as such, but which is not identical to Eta-1/osteopontin. In one embodiment, an IL-10 inhibitory component is a polypeptide which is derived from Eta-1/osteopontin. Accordingly, the IL-10 inhibitory component has features of Eta-1/osteopontin (*e.g.*, functions to inhibit IL-10 secretion) but is not identical to osteopontin. In one embodiment, an IL-10 inhibitory component includes a polypeptide which has at least 50% identity to an IL-10 inhibitory domain of Eta-1/osteopontin. In yet another embodiment, an IL-10 inhibitory component is at least 55%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or more identical to an IL-10 inhibitory domain of Eta-1/osteopontin IL-10. In yet another embodiment, an IL-10 inhibitory component includes a polypeptide which has at least 80-85%, 85-90%, 90-95%, 96%, 97%, 98%, 99% or more identity to about amino acids 169-266 of SEQ ID NO:2 In another embodiment, an IL-10 inhibitory component includes a polypeptide which is at least 65-160 amino acids in length. In another embodiment, an IL-10 inhibitory component includes a polypeptide which is between 30-35, 40-45, 45-50, 50-55, 55-60, 60-65, 65-70, 70-75, 75-80, 80-85, 85-90 90-100 or more amino acids in length. In another embodiment, an IL-10 inhibitory component includes a polypeptide which is greater than 115 amino acids in length.

Another embodiment of the present invention features biosynthetic molecules which include an IL-10 inhibitory component having an amino acid sequence sufficiently homologous to the an IL-10 inhibitory domain of a protein having the amino acid sequence of Eta-1/osteopontin (*e.g.*, SEQ ID NO:2), as defined herein. In a preferred embodiment, an IL-10 inhibitory component retains an IL-10 inhibitory, preferably an IL-10 inhibitory activity of Eta-1/osteopontin. In another embodiment, a molecule has an immunomodulatory activity. In another embodiment, an IL-10 inhibitory component includes an amino acid sequence selected from the group

consisting of amino acids 71-180 of SEQ ID NO:4, amino acids 58-166 of SEQ ID NO:6, or amino acids 44-153 of SEQ ID NO:8.

The present invention further features isolated nucleic acid molecules which encode the biosynthetic immunomodulatory molecules of the present invention. In one embodiment, an isolated nucleic acid molecule of the present invention includes a nucleic acid sequence which encodes an IL-10 inhibitory domain. In another embodiment, an isolated nucleic acid molecule of the present invention includes a nucleic acid sequence which encodes a biomodulatory domain. In another embodiment, an isolated nucleic acid molecule of the present invention includes a nucleic acid sequence (SEQ ID NO:9) which encodes Immunomodulin-1 (SEQ ID NO:10).

A. Isolated Biosynthetic Molecules

"Isolated" or "purified" biosynthetic molecules are also features according to the present invention. "Isolated" or "purified" biosynthetic molecules are substantially free of cellular material or other contaminating proteins from the cell or tissue source from which the molecule is derived, or substantially free from chemical precursors or other chemicals when chemically synthesized. The phrases "substantially free of cellular material" and "substantially free of chemical precursors or other chemicals" are as defined herein for isolated Eta-1/proteins or polypeptides.

Also featured are isolated nucleic acid molecules encoding the biosynthetic molecules of the present invention, vectors including such nucleic acid molecules, as well as host cells into which such vectors have been incorporated, as defined herein. Also featured are methods of making the biosynthetic molecules of the present invention, as described herein for making Eta-1/osteopontin proteins or polypeptides.

Biologically active portions of a biosynthetic molecules of the present invention are also featured and include molecules sufficiently homologous to or derived from the biosynthetic molecules of the present invention which include less amino acids than the full biosynthetic molecules, and exhibit at least one activity of the biosynthetic molecules. Typically, biologically active portions at least one domain or motif with at least one activity of the biosynthetic molecules. A biologically active portion can be a polypeptide which is, for example, 10, 25, 50, 100 or more amino acids in length.

III. Pharmaceutical Compositions

The nucleic acid molecules, proteins, and biosynthetic molecules (also referred to herein as "active compounds") of the invention can be incorporated into pharmaceutical compositions suitable for administration. Such compositions typically comprise the nucleic acid molecule, protein, or antibody and a pharmaceutically acceptable carrier. As used herein the language "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. Examples of routes of administration include parenteral, e.g., intravenous, intradermal, subcutaneous, oral (e.g., inhalation), transdermal (topical), transmucosal, and rectal administration. Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic

water, Cremophor EL™ (BASF, Parsippany, NJ) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyetheylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating the active compound (e.g., a Immunomodulin protein or anti-Immunomodulin antibody) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Oral compositions generally include an inert diluent or an edible carrier. They can be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches, or capsules. Oral compositions can also be prepared using a fluid carrier for use as a mouthwash, wherein the compound in the fluid carrier is applied orally and swished and expectorated or swallowed. Pharmaceutically

compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

For administration by inhalation, the compounds are delivered in the form of an aerosol spray from pressured container or dispenser which contains a suitable propellant, e.g., a gas such as carbon dioxide, or a nebulizer.

Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active compounds are formulated into ointments, salves, gels, or creams as generally known in the art.

The compounds can also be prepared in the form of suppositories (e.g., with conventional suppository bases such as cocoa butter and other glycerides) or retention enemas for rectal delivery.

In one embodiment, the active compounds are prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically

acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Patent No. 4,522,811.

It is especially advantageous to formulate oral or parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and the limitations inherent in the art of compounding such an active compound for the treatment of individuals.

Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD50/ED50. Compounds which exhibit large therapeutic indices are preferred. While compounds that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the method of the invention, a "therapeutically effective" dose can be estimated initially from cell culture assays. A "therapeutically effective" dose can be further formulated in animal models to achieve a circulating plasma concentration range that includes the IC50 (i.e., the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

The nucleic acid molecules of the invention can be inserted into vectors and used as gene therapy vectors. Gene therapy vectors can be delivered to a subject by, for example, intravenous injection, local administration (see U.S. Patent 5,328,470) or by stereotactic injection (see e.g., Chen et al. (1994) *PNAS* 91:3054-3057). The pharmaceutical preparation of the gene therapy vector can include the gene therapy vector in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery vector can be produced intact from recombinant cells, e.g., retroviral vectors, the pharmaceutical preparation can include one or more cells which produce the gene delivery system.

The pharmaceutical compositions can be included in a container, pack, or dispenser together with instructions for administration.

IV. Uses and Methods Featuring Biosynthetic Immunomodulatory Molecules of the Present invention

A. Assays

The ability of an stimulatory or inhibitory agent of the invention (*e.g.*, an Eta-1/osteopontin modulator or a biosynthetic immunomodulatory molecule) to modulate an immune response (*e.g.*, to bias an immune response from a type-1 to a type-2 immune response or from a type-2 to a type-1 immune response) can be evaluated using an *in vitro* culture system such as that described herein in the Examples. For example, expression, production or secretion of a cytokine can be determined (*e.g.*, of a type-1 or a type-2 cytokine) or a cytokine profile can be determined (*e.g.*, a type-1 or a type-2 cytokine profile). Immune effector cells (*e.g.*, peripheral blood mononuclear cells) can be cultured in the presence of an stimulatory or inhibitory agent of the invention as described in the examples in a medium suitable for culture of the chosen cells. In the case of assaying for the ability of an inhibitory agent of the invention to modulate an immune response (*e.g.*, an Eta-1/osteopontin inhibitory modulator or an IL-10 component-containing biosynthetic immunomodulatory molecule) it may be necessary to also stimulate cells with a known stimulatory agent. After a period of time (*e.g.*, 24-72 hours), production of cytokine(s) (*e.g.*, at least one type-1 cytokine, at least one type-2 cytokine, a type-1 cytokine profile or a type-2 cytokine profile) is assessed

by determining the level of the cytokine in the culture supernatant as described in the examples. The ability of a stimulatory agent to stimulate cytokine production is evidenced by a higher level of cytokine in the supernatants of cells cultured in the presence of the stimulatory agent compared to the level of cytokine in the supernatant of cells cultured alone or in the presence of a control. The ability of an inhibitory agent to inhibit cytokine production is evidenced by a lower level of cytokine in the supernatants of cells cultured in the presence of both the inhibitory agent and the stimulatory agent compared to the level of cytokine in the supernatant of cells cultured only in the presence of the stimulatory agent.

B. Therapeutic Uses

The present invention provides for both prophylactic and therapeutic methods of treating subjects (*e.g.*, human subjects). In one aspect, the invention provides a method for preventing or treating a disease or a disorder in a subject prophylactically or therapeutically. Administration of a agent prophylactically can occur prior to the manifestation of symptoms of an undesired disease or disorder, such that the disease or disorder is prevented or, alternatively, delayed in its progression. The prophylactic methods of the present invention can be carried out in a similar manner to therapeutic methods described herein, although dosage and treatment regimes may differ.

Another aspect of the invention pertains to methods for treating a subject therapeutically. In one embodiment, the present invention includes methods of modulating an immune response. In particular, modulation of an immune response includes, but is not limited to, modulation of cellular toxicity, modulation of cytokine expression, production or secretion (*e.g.*, enhancement or inhibition of cytokine expression, production or secretion). A preferred embodiment of the invention involves modulation of IL-12, in particular, stimulation of IL-12 using an Eta-1/osteopontin stimulatory modulator or, alternatively, inhibition of IL-12 using an Eta-1/osteopontin inhibitory modulator. Another preferred embodiment of the invention involves modulation of IL-10, in particular, inhibition of IL-10 using an Eta-1/osteopontin stimulatory modulator or, alternatively, stimulation of IL-10 using an Eta-1/osteopontin inhibitory modulator. Accordingly, the present method has therapeutic utility in biasing

an immune response towards, or away from, a type-1 immune response depending upon the desired therapeutic regimen. Such modulatory methods are particularly useful in diseases such as cancer, in immunology, for example, in allergy, organ transplantation and organ rejection. Moreover, the immunomodulatory methods of the present invention can be used to treat an immunocompromized individual to enhance immunity. Uses to increase resistance to viral infection and enhance the rejection of foreign molecules are also within the scope of the present invention. The immunomodulatory methods of the present invention are further useful in wound healing. For example, an enhancement of type-1 immunity in a burn victim, or alternatively, at the burn or wound site, can result in a more rapid immune response, thus preventing infection. The immunomodulatory methods of the present invention are further useful in treating asthma. These various immunomodulatory therapeutic applications are described in further detail in the following subsection.

V. Clinical Applications of the Modulatory Methods of the Invention

The identification of Eta-1/osteopontin as a critical regulator of type 1 immunity allows for selective manipulation of T cell subsets in a variety of clinical situations using the modulatory methods of the invention. The stimulatory methods of the invention (*i.e.*, methods that use an Eta-1/osteopontin stimulatory agent) upregulate the production of the Th1-associated cytokine IL-12 and/or dowregulate the production of the Th2-associated cytokine IL-10, with concomitant promotion of a type 1 immune responses and downregulation of type 2 immune responses. In contrast, the inhibitory methods of the invention (*i.e.*, methods that use an Eta-1/osteopontin inhibitory agent) downregulate the production of the Th1-associated cytokine IL-12 and/or upregulate the production of the Th2-associated cytokine IL-10, with concomitant downregulation of a type 1 immune responses and promotion of type 2 immune responses.

Thus, to treat a disease condition wherein a type 1 immune response is beneficial, a stimulatory method of the invention is selected such that type 1 immune responses are promoted while downregulating type 2 immune responses. Alternatively, to treat a disease condition wherein a type 2 immune response is beneficial, an inhibitory method of the invention is selected such that type 1 immune responses are downregulated while promoting type 2 immune responses. Application of the methods

of the invention to the treatment of disease conditions may result in cure of the condition, a decrease in the type or number of symptoms associated with the condition, either in the long term or short term (*i.e.*, amelioration of the condition) or simply a transient beneficial effect to the subject.

Numerous disease conditions associated with a predominant type 1 or type immune responses have been identified and could benefit from modulation of the type of response mounted in the individual suffering from the disease condition. Application of the immunomodulatory methods of the invention to such diseases is described in further detail below.

A. *Allergies*

Allergies are mediated through IgE antibodies whose production is regulated by the activity of Th2 cells and the cytokines produced thereby. In allergic reactions, IL-4 is produced by Th2 cells, which further stimulates production of IgE antibodies and activation of cells that mediate allergic reactions, *i.e.*, mast cells and basophils. IL-4 also plays an important role in eosinophil mediated inflammatory reactions. Accordingly, the Eta-1/osteopontin stimulatory methods of the invention, which promote type 1 responses and inhibit type 2 responses, can be used to inhibit the type 2 responses (e.g., production of Th2-associated cytokines) in allergic patients as a means to downregulate production of pathogenic IgE antibodies. A stimulatory agent may be directly administered to the subject or cells (*e.g.*, Th0 cells or Th1 cells) may be obtained from the subject, contacted with a stimulatory agent *ex vivo*, and readministered to the subject. Moreover, in certain situations it may be beneficial to coadminister to the subject the allergen together with the stimulatory agent or cells treated with the stimulatory agent to inhibit (*e.g.*, desensitize) the allergen-specific type 2 response. The treatment may be further enhanced by administering other Th1-promoting agents, such as the cytokine IL-12 or antibodies to Th2-associated cytokines (*e.g.*, anti-IL-4 or anti-IL-10 antibodies), to the allergic subject in amounts sufficient to further stimulate a type 1 immune response.

B. *Cancer*

The expression of Th2-promoting cytokines has been reported to be elevated in cancer patients (see *e.g.*, Yamamura, M., *et al.* (1993) *J. Clin. Invest.* 91:1005-1010; Pisa, P., *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:7708-7712) and malignant disease is often associated with a shift from Th1 type responses to Th2 type responses along with a worsening of the course of the disease. Accordingly, the stimulatory methods of the invention can be used to promote type 1 responses and inhibit type 2 responses (e.g., the production of Th2-associated cytokines) in cancer patients, as a means to counteract the Th1 to Th2 shift and thereby promote an ongoing Th1 response in the patients to ameliorate the course of the disease. The stimulatory methods can involve either direct administration of a stimulatory agent to a subject with cancer or *ex vivo* treatment of cells obtained from the subject (*e.g.*, Th0 or Th1 cells) with a stimulatory agent followed by readministration of the cells to the subject. The treatment may be further enhanced by administering other Th1-promoting agents, such as the cytokine IL-12 or antibodies to Th2-associated cytokines (*e.g.*, anti-IL-4 or anti-IL-10 antibodies), to the recipient in amounts sufficient to further stimulate a Th1-type response.

C. *Infectious Diseases (e.g., Bacterial or Viral)*

The expression of Th2-promoting cytokines also has been reported to increase during a variety of infectious diseases (including viral and bacterial infectious diseases), including HIV infection, tuberculosis, leishmaniasis, schistosomiasis, filarial nematode infection and intestinal nematode infection (see *e.g.*; Shearer, G.M. and Clerici, M. (1992) *Prog. Chem. Immunol.* 54:21-43; Clerici, M and Shearer, G.M. (1993) *Immunology Today* 14:107-111; Fauci, A.S. (1988) *Science* 239:617-623; Locksley, R. M. and Scott, P. (1992) *Immunoparasitology Today* 1:A58-A61; Pearce, E.J., *et al.* (1991) *J. Exp. Med.* 173:159-166; Grzych, J-M., *et al.* (1991) *J. Immunol.* 141:1322-1327; Kullberg, M.C., *et al.* (1992) *J. Immunol.* 148:3264-3270; Bancroft, A.J., *et al.* (1993) *J. Immunol.* 150:1395-1402; Pearlman, E., *et al.* (1993) *Infect. Immun.* 61:1105-1112; Else, K.J., *et al.* (1994) *J. Exp. Med.* 179:347-351) and such infectious diseases are also associated with a Th1 to Th2 shift in the immune response.

Accordingly, the stimulatory methods of the invention can be used in infectious diseases (caused by bacterial, viral or other pathogenic origins) to promote a type 1 response and inhibit a type 2 response (e.g., the production of Th2-associated cytokines) in subjects with infectious diseases, as a means to counteract the Th1 to Th2 shift and thereby promote an ongoing Th1 response in the patients to ameliorate the course of the infection. The stimulatory method can involve either direct administration of a stimulatory agent to a subject with an infectious disease or *ex vivo* treatment of cells obtained from the subject (*e.g.*, Th0 or Th1 cells) with a stimulatory agent followed by readministration of the cells to the subject. The treatment may be further enhanced by administering other Th1-promoting agents, such as the cytokine IL-12 or antibodies to Th2-associated cytokines (*e.g.*, anti-IL-4 or anti-IL-10 antibodies), to the recipient in amounts sufficient to further stimulate a Th1-type response.

D. *Autoimmune Diseases*

The Eta-1/osteopontin inhibitory methods of the invention can be used therapeutically in the treatment of autoimmune diseases that are associated with a Th2-type dysfunction. Many autoimmune disorders are the result of inappropriate activation of T cells that are reactive against self tissue and that promote the production of cytokines and autoantibodies involved in the pathology of the diseases. Modulation of T helper-type responses can have an effect on the course of the autoimmune disease. For example, in experimental allergic encephalomyelitis (EAE), stimulation of a Th2-type response by administration of IL-4 at the time of the induction of the disease diminishes the intensity of the autoimmune disease (Paul, W.E., *et al.* (1994) *Cell* 76:241-251). Furthermore, recovery of the animals from the disease has been shown to be associated with an increase in a Th2-type response as evidenced by an increase of Th2-specific cytokines (Koury, S. J., *et al.* (1992) *J. Exp. Med.* 176:1355-1364). Moreover, T cells that can suppress EAE secrete Th2-specific cytokines (Chen, C., *et al.* (1994) *Immunity* 1:147-154). Since stimulation of a Th2-type response in EAE has a protective effect against the disease, stimulation of a Th2 response in subjects with multiple sclerosis (for which EAE is a model) is likely to be beneficial therapeutically.

Similarly, stimulation of a Th2-type response in type I diabetes in mice provides a protective effect against the disease. Indeed, treatment of NOD mice with IL-4 (which promotes a Th2 response) prevents or delays onset of type I diabetes that normally develops in these mice (Rapoport, M.J., *et al.* (1993) *J. Exp. Med.* 178:87-99). Thus, stimulation of a Th2 response in a subject suffering from or susceptible to diabetes may ameliorate the effects of the disease or inhibit the onset of the disease.

Yet another autoimmune disease in which stimulation of a Th2-type response may be beneficial is rheumatoid arthritis (RA). Studies have shown that patients with rheumatoid arthritis have predominantly Th1 cells in synovial tissue (Simon, A.K., *et al.*, (1994) *Proc. Natl. Acad. Sci. USA* 91:8562-8566). By stimulating a Th2 response in a subject with RA, the detrimental Th1 response can be concomitantly downmodulated to thereby ameliorate the effects of the disease.

Accordingly, the Eta-1/osteopontin inhibitory methods of the invention, which downregulate type 1 responses (e.g., by inhibition of IL-12 production) can be used to shift the immune response to a type 2 immune response (e.g., stimulating production of Th2-associated cytokines) in subjects suffering from, or susceptible to, an autoimmune disease in which a Th2-type response is beneficial to the course of the disease. The inhibitory method can involve either direct administration of an inhibitory agent to the subject or *ex vivo* treatment of cells obtained from the subject with an inhibitory agent followed by readministration of the cells to the subject. The treatment may be further enhanced by administering other Th2-promoting agents, such as IL-4 or IL-10 itself or antibodies to Th1-associated cytokines (e.g., anti-IL-12 antibodies) to the subject in amounts sufficient to further stimulate a Th2-type response.

In contrast to the autoimmune diseases described above in which a Th2 response is desirable, other autoimmune diseases may be ameliorated by a Th1-type response. Such diseases can be treated using an Eta-1/osteopontin stimulatory agent of the invention (as described above for cancer and infectious diseases). The treatment may be further enhanced by administrating a Th1-promoting cytokine (*e.g.*, IFN-γ) to the subject in amounts sufficient to further stimulate a Th1-type response.

The efficacy of agents for treating autoimmune diseases can be tested in the above described animal models of human diseases (*e.g.*, EAE as a model of multiple sclerosis and the NOD mice as a model for diabetes) or other well characterized animal

models of human autoimmune diseases. Such animal models include the *mrl/lpr/lpr* mouse as a model for lupus erythematosus, murine collagen-induced arthritis as a model for rheumatoid arthritis, and murine experimental myasthenia gravis (see Paul ed., *Fundamental Immunology*, Raven Press, New York, 1989, pp. 840-856). A modulatory (*i.e.*, stimulatory or inhibitory) agent of the invention is administered to test animals and the course of the disease in the test animals is then monitored by the standard methods for the particular model being used. Effectiveness of the modulatory agent is evidenced by amelioration of the disease condition in animals treated with the agent as compared to untreated animals (or animals treated with a control agent).

Non-limiting examples of autoimmune diseases and disorders having an autoimmune component that may be treated according to the invention include diabetes mellitus, arthritis (including rheumatoid arthritis, juvenile rheumatoid arthritis, osteoarthritis, psoriatic arthritis, bacterial arthritis), multiple sclerosis, myasthenia gravis, systemic lupus erythematosis, autoimmune thyroiditis, dermatitis (including atopic dermatitis and eczematous dermatitis), psoriasis, Sjögren's Syndrome, including keratoconjunctivitis sicca secondary to Sjögren's Syndrome, alopecia areata, allergic responses due to arthropod bite reactions, Crohn's disease, aphthous ulcer, iritis, conjunctivitis, keratoconjunctivitis, ulcerative colitis, asthma, allergic asthma, cutaneous lupus erythematosus, scleroderma, vaginitis, proctitis, drug eruptions, leprosy reversal reactions, erythema nodosum leprosum, autoimmune uveitis, allergic encephalomyelitis, acute necrotizing hemorrhagic encephalopathy, idiopathic bilateral progressive sensorineural hearing loss, aplastic anemia, pure red cell anemia, idiopathic thrombocytopenia, polychondritis, Wegener's granulomatosis, chronic active hepatitis, Stevens-Johnson syndrome, idiopathic sprue, lichen planus, Crohn's disease, Graves ophthalmopathy, sarcoidosis, primary biliary cirrhosis, uveitis posterior, and interstitial lung fibrosis.

E. *Transplantation Rejection*

While graft rejection or graft acceptance may not be attributable exclusively to the action of a particular T cell subset (*i.e.*, Th1 or Th2 cells) in the graft recipient (for a discussion see Dallman, M.J. (1995) *Curr. Opin. Immunol.* 7:632-638), numerous studies have implicated a predominant Th2 response in prolonged graft

survival or a predominant Th1 response in graft rejection. For example, graft acceptance has been associated with production of a Th2 cytokine pattern and/or graft rejection has been associated with production of a Th1 cytokine pattern (see *e.g.*, Takeuchi, T. *et al.* (1992) *Transplantation* 53:1281-1291; Tzakis, A.G. *et al.* (1994) *J. Pediatr. Surg.* 29:754-756; Thai, N.L. *et al.* (1995) *Transplantation* 59:274-281). Additionally, adoptive transfer of cells having a Th2 cytokine phenotype prolongs skin graft survival (Maeda, H. *et al.* (1994) *Int. Immunol.* 6:855-862) and reduces graft-versus-host disease (Fowler, D.H. *et al.* (1994) *Blood* 84:3540-3549; Fowler, D.H. *et al.* (1994) *Prog. Clin. Biol. Res.* 389:533-540). Still further, administration of IL-4, which promotes Th2 differentiation, prolongs cardiac allograft survival (Levy, A.E. and Alexander, J.W. (1995) *Transplantation* 60:405-406), whereas administration of IL-12 in combination with anti-IL-10 antibodies, which promotes Th1 differentiation, enhances skin allograft rejection (Gorczynski, R.M. *et al.* (1995) *Transplantation* 60:1337-1341).

Accordingly, the Eta-1/osteopontin inhibitory methods of the invention, which inhibit type 1 immune responses, can be used to shift the bias toward type 2 immune responses in transplant recipients to prolong survival of the graft. The inhibitory methods can be used both in solid organ transplantation and in bone marrow transplantation (*e.g.*, to inhibit graft-versus-host disease). The inhibitory method can involve either direct administration of an inhibitory agent to the transplant recipient or *ex vivo* treatment of cells obtained from the subject with an inhibitory agent followed by readministration of the cells to the subject. The treatment may be further enhanced by administering other Th2-promoting agents, such asIL-4 or IL-10 itself or antibodies to Th1-associated cytokines (e.g., anti-IL-12 antibodies), to the recipient in amounts sufficient to further stimulate a Th2-type response.

F. *Other Disorders for Upregulation of Type 1 Immune Responses*

In addition to the foregoing, there are numerous other disorders in which it can be beneficial to upregulate (i.e., bias toward) type 1 immune responses using the Eta-1/osteopontin stimulatory methods of the invention, as follows:

Burn associated sepsis is associated with the excess production of the type 2 cytokine IL-10. Accordingly, use of an Eta-1/osteopontin stimulatory method of

the invention to promote type 1 responses (e.g., by upregulating IL-12 production and/or downregulating IL-10 production) can be beneficial in the treatment of burn-associated sepsis.

Immunodeficiency disorders often are associated with a lack of, or insufficient, type 1 immunity. Accordingly, immunodeficiency disorders such as AIDS, bone marrow transplant-associated immunodeficiency, and chemotherapy-associated immunodeficiencies, can be treated using an Eta-1/osteopontin stimulatory method of the invention to promote type 1 responses (e.g., by upregulating IL-12 production and/or downregulating IL-10 production).

Any of the foregoing treatments may be further enhanced by administering other Th1-promoting agents, such as the cytokine IL-12 or antibodies to Th2-associated cytokines (*e.g.*, anti-IL-4 or anti-IL-10 antibodies), to the recipient in amounts sufficient to further stimulate a Th1-type response.

G. *Other Disorders for Downregulation of Type 1 Immune Responses*

In addition to the foregoing, there are numerous other disorders in which it can be beneficial to downregulate (i.e., bias away from) type 1 immune responses (and bias toward type 2 immune responses) using the Eta-1/osteopontin inhibitory methods of the invention, as follows:

Granulomatous disorders result from excessive type 1 responses (discussed further in Example 1) and experiments have demonstrated that in the absence of Eta-1 (e.g., in an Eta-1 deficient animal) sarcoid-type granulomas fail to form. Accordingly, use of an Eta-1/osteopontin inhibitory method of the invention to downregulate type 1 responses (e.g., by downregulating IL-12 production and/or upregulating IL-10 production) can be beneficial in the treatment of granulomatous disorders.

Herpes Simplex Virus Keratitis (HSK) results from corneal infection by Herpes Simplex Virus-1 (HSV-1) that leads to a destructive autoimmune inflammatory reaction that depends on the production of IL-12 and that is inhibited by IL-10 (discussed further in Example 2). Accordingly, use of an Eta-1/osteopontin inhibitory method of the invention to downregulate type 1 responses (e.g., by downregulating IL-

12 production and/or upregulating IL-10 production) can be beneficial in the treatment of HSK.

Bacterial arthritis is associated with excessive type 1 responses subsequent to bacterial infection. Accordingly, use of an Eta-1/osteopontin inhibitory method of the invention to downregulate type 1 responses (e.g., by downregulating IL-12 production and/or upregulating IL-10 production) can be beneficial in the treatment of bacterial arthritis.

Any of the foregoing treatments may be further enhanced by administering other Th2-promoting agents, such asIL-4 or IL-10 itself or antibodies to Th1-associated cytokines (e.g., anti-IL-12 antibodies), to the recipient in amounts sufficient to further promote a Th2-type response.

In addition to the foregoing disease situations, the modulatory methods of the invention also are useful for other purposes. For example, the stimulatory methods of the invention (*i.e.*, methods using a stimulatory agent) can be used to stimulate production of Th1-promoting cytokines (*e.g.*, IL-12) *in vitro* for commercial production of these cytokines (*e.g.*, cells can be contacted with the stimulatory agent *in vitro* to stimulate IL-12 production and the IL-12 can be recovered from the culture supernatant, further purified if necessary, and packaged for commercial use).

Furthermore, the modulatory methods of the invention can be applied to vaccinations to promote either a Th1 or a Th2 response to an antigen of interest in a subject. That is, the agents of the invention can serve as adjuvants to direct an immune response to a vaccine either to a Th1 response or a Th2 response. For example, to stimulate an antibody response to an antigen of interest (*i.e.*, for vaccination purposes), the antigen and an Eta-1 inhibitory agent of the invention can be coadministered to a subject to bias the response towards type 2 responses (e.g., antibody production) to the antigen in the subject, since Th2 responses provide efficient B cell help and promote IgG1 production. Alternatively, to promote a cellular immune response to an antigen of interest, the antigen and a stimulatory agent of the invention can be coadministered to a subject to promote a Th1 response to the antigen in a subject, since Th1 responses favor the development of cell-mediated immune responses (*e.g.*, delayed hypersensitivity responses). The antigen of interest and the modulatory agent can be formulated together

into a single pharmaceutical composition or in separate compositions. In a preferred embodiment, the antigen of interest and the modulatory agent are administered simultaneously to the subject. Alternatively, in certain situations it may be desirable to administer the antigen first and then the modulatory agent or vice versa (for example, in the case of an antigen that naturally evokes a Th2 response, it may be beneficial to first administer the antigen alone to stimulate a Th2 response and then administer an Eta-1/osteopontin stimulatory agent, alone or together with a boost of antigen, to shift the immune response to a Th1 response).

VI. Tumor Immunity, Irradiated Tumor Cells

The present invention also features methods of modulating tumor immunity. Such methods are based, at least in part, on the understanding that tumor cells are capable of escaping destruction by a subject's immune system, *i.e.*, are capable of escaping the subject's natural immune responses. Accordingly, in one embodiment, the invention features a method of modulating tumor immunity which involves contacting a tumor cell with an Eta-1/osteopontin modulator such that tumor immunity is modulated. A preferred embodiment features a method of enhancing a type 1 response to a tumor cell which involves contacting the cell with an Eta-1 stimulatory agent such that a type 1 response against the cell is enhanced. Another preferred embodiment features a method of enhancing a type 1 response to a tumor cell which involves contacting the cell with an Eta-1 stimulatory agent such that a type 1 response against the cell is stimulated (*e.g.*, is stimulated by the tumor cell so contacted). In one embodiment, the tumor cell is contacted *in vivo*. In another embodiment, the tumor cell is contacted *ex vivo*. For example, tumor cells can be isolated from the subject and cultures in the presence of an Eta-1/osteopontin modulator (*e.g.*, an Eta-1 stimulatory agent). In another embodiment, the method can further include administering (*e.g.*, readministering) the cells to the patient. In yet another embodiment, the cells are transfected in culture with an isolated nucleic acid Eta-1/osteopontin modulator (*e.g.*, a nucleic acid molecule encoding Eta-1/osteopontin or a biologically active fragment thereof, or encoding a biosynthetic molecule of the present invention. Methods for transfecting cells, vectors and the like are as described herein (for example, in section I.B.)

In yet another embodiment. the invention features methods of modulating tumor immunity (*e.g.*, methods of enhancing a type 1 response against a tumor or tumor cells) which further includes the step of irradiating the tumor cells (*e.g.*, before or after contacting with an Eta-1 modulator) such that the cells are incapable of replicating once administered to the patient. In yet another embodiment, the method further features the step of transfecting the cells with GMCSF. Also featured are tumor cells treated with an Eta-1/osteopontin modulator of the present invention. In one embodiemnt, the invention features tumor cells transfected with an Eta-1-encoding nucleic acid molecule or nucleic acid molecule encoding a biologically active fragment of Eta-1/osteopontin. In another embodiment, the invention features tumor cells transfected with Eta-1/osteopontin and GMCSF. In yet another embodiemnt, the invention features a tumor cells transfected with a nucleic acid molecule which encodes a biosynthetic immunomodulatory molecule of the preset invention.

The present invention is further illustrated by the following Examples which in no way should be construed as further limiting. The entire contents of all of the references (including literature references, issued patents, and published patent applications) cited throughout this application are hereby expressly incorporated by reference.

Exemplification

Examples 1-4 demonstrate an essential role for Eta-1/osteopontin in regulating immune responses (*e.g.*, type-1 immune responses) *in vivo*. These examples further demonstrate the applicability of administering Eta-1/osteopontin as an *in vivo* approach to regulating such immune responses.

Example 1: Eta-1/opn-dependent modulation of type-1 immunity (*e.g.*, in a classical granulomatous response) *in vivo* in control, nude, cytokine-deficient and Eta-1/opn-deficient mice

An early and essential step in type-1 immunity is the migration of macrophages/dendritic cells to the site of infection, and subsequent activation of the recruited macrophages, a process that is controlled by CD4 T-cells. Eta-1/osteopontin is the most abundantly expressed mRNA transcript after activation of CD4 cells (Patarca *et al.* (1989) *J. Exp. Med.* 170:145-161; Weber *et al.* (1997) *Proc. Assoc. Am. Physicians* 109:1-9; Rittling and Denhardt (1999) *Exp. Nephrol.* 7:103). Production of IL-12 by activated macrophages/dendritic cells and reception of the IL-12 signal by CD4 cells are subsequent critical steps in this process. Although an interaction between CD40 ligand on activated T-cells and CD40 on macrophages can induce IL-12 expression (Scheicher *et al.* (1995) *Eur. J. Immunol.* 25, 1566; Macatonia *et al.* (1995) *J. Immunol.* 154:5071; Murphy (1998) *Curr. Opin. Immunol.* 10:226) this interaction also induces the inhibitory IL-10 cytokine and may not suffice for induction of IL-12 *in vitro* (Ria *et al.* (1998) *Eur. J. Immunol.* 28:2003) or for sustained levels of IL-12 that follow viral infection *in vivo* (Sharma *et al.* (1998) *J. Immunol.* 161:5357).

The Eta-1 gene is expressed in T cells early in the course of bacterial infections (within 48 hours), and interaction of its protein product with macrophages can induce inflammatory responses (Singh *et al.* (1990) *Exp. Med.* 171:1931; Yu *et al.* (1998) *Proc. Assoc. Am. Physicians* 110:50; Denhardt and Noda (1998) *J. Cell. Biochem. Suppl.* 30/31:92). Genetic resistance to infection by certain strains of Rickettsia may depend on Eta-1-dependent attraction of monocytes into infectious sites and acquisition of bacteriocidal activity (Patarca *et al.* (1993) *Crit. Rev. Immunol.* 13:225; Jerrells and Osterman (1981) *Infect. Immun.* 31:1014); the granulomatous responses characteristic of sarcoidosis and tuberculosis are associated with high levels of Eta-1 expression (Nau *et al.*(1997) *Proc. Natl. Acad. Sci. U.S.A.* 94:6414; O'Regan *et al.* (1999) *J. Immunol.* 162:1024).

Granuloma formation in these human diseases is a cellular consequence of type-1 immunity (Patarca *et al.* (1993) *Crit. Rev. Immunol.* 13:225; Jerrells and Osterman (1981) *Infect. Immun.* 31:1014). Accordingly, a valuable *in vivo* animal model for studying type-1 immune responses involves inducing sarcoid-type granulomas in mice

by injection of polyvinyl pyrrolidone (PVP) (van den Bogert *et al.* (1986) *Virchows Arch.* 51:39). Because certain murine models of parasite-induced granulomas may reflect a mixture of type-2 and type-1 immunity (O'Garra (1998) *Immunity* 8:275), the importance of IL-12-dependent type-1 immunity in this murine model of granuloma formation was first established.

The granulomatous response was first measured in control (C57BL/6 (+/+)) and nude (C57BL/6 *nu/nu*) mice. PVP-dependent granulomas were formed by injecting mice subcutaneously above the right hind limb with 500 µl of 0.5% PVP. After 5 days, mice were killed, and tissue was extracted for histologic analysis. Figure 1A depicts the data as (1) the mean number of granulomas per high-power field (HPF) (X200 magnification); (2) as the mean number of cells per granuloma after examination of 50 to 80 HPF per mouse; and (3) as the product of these two indices, termed "granuloma burden". (Error bars indicate 1 SEM.). An intense granulomatous response was provoked shortly after subcutaneous injection of PVP into C57BL/6 (+/+) but not C57BL/6 *nu/nu* strains of mice (see *e.g.*, top two bars of Figure 1A).

It was next determined whether Eta-1 administration could reconstitute the granulomatous response in C57BL/6 *nu/nu* strains of mice. For experiments using purified Eta-1/opn to modulate immunity, Eta-1 is prepared as follows. To generate naturally-produced (native) Eta-1/opn, MC3T3E1 cells or Ar5v T-cells were grown in defined media (consisting of DME/H12 supplemented with pyruvate, insulin, transferrin, selenium and ethanolmine) in 5% CO_2 at 37°C. Media was dialized against PBS and concentrated using a Millipore tangential flow system applied to Millipore LC100 equipped with a DEAE-Memsep 1000 cartridge and developed in a discontinuous gradient of 0 to 1 M NaCl in phosphate buffer, pH 7.4. Eta-1/opn-containing fractions were pooled (the major Eta-1/opn peak eluted at 0.26 M salt), concentrated by ultrafiltration, chromatofocused on mono P columns (Pharmacia) at pH 8.2, developed with polybuffer 74 (Pharmacia) and the major Eta-1/opn fraction eluted from monobeads at pH 4.6. The protein was judged pure by several criteria including SDS electrophoresis and amino acid sequence analysis (both N-terminal and internal peptide analysis). Mass spectroscopic analysis revealed a peak centered around a mass of 35,400 that was highly phosphorylated (11 mols of phosphate/mol of protein), O-glycosylation but not N-glycosylation, and no measurable sulfate. For

dephosphorylated, naturally-produced Eta-1/opn. 5 mg of purified Eta-1/opn was incubated with 6 units (60 units/mg) type II potato acid phosphatase in 20 mM phosphate buffer pH 4.8 at 37°C for 2 h. After adjusting the pH to 8.2, dephosphorylated protein was applied to a chromatofocusing column and the major peak eluted at a pH of 5.1; amino acid analysis of the protein revealed a phosphate content of less than 1 mol/mol protein.

When C57BL/6 *nu/nu* mice were coinjected with PVP inoculum plus 10 µg of Eta-1, the granulomatous response was partially restored, demonstrating that the Eta-1/opn gene product can partially substitute for activated T lymphocytes in this setting. The granulomatous response was likewise determined in cytokine-deficient (C57BL/6 IL-$12^{-/-}$ and C57BL/6 IL-$10^{-/-}$) mice. The granulomatous response was diminished by 70 to 80% in C57BL/6 IL-$12^{-/-}$ mice and was enhanced two- to three-fold in C57BL/6 IL-$10^{-/-}$ mice.

It was then asked whether mice deficient in Eta-1 secondary to targeted gene mutation formed granulomas after PVP injection. C57BL/6 × 129/SV Eta-$1^{-/-}$ mice generated as described by Rittling *et al.* (1998) *J. Bone Miner. Res.* 13:1101, were compared to either Eta-$1^{+/+}$ littermates or age-matched C57BL/6 × 129/SV mice as controls. Histological analysis was performed on tissue sections from PVP injection sites. Briefly, samples were fixed in 10% buffered formalin and embedded in paraffin. Embedded sampled were sectioned into 4- to 5-µm serial sections and stained with hematoxylin and eosin. Images were captured with a Sony DXC-970MD video camera and Optima 5.2 Histomorphometric analysis software.

Histological analysis of tissue sections at PVP injection sites at 20X, 100X and 400X magnifications showed granulomatous infiltrations of mononuclear cells in subcutaneous dermal and subdermal areas in Eta-$1^{+/+}$ mice 5 days after injection of PVP, PBS, or PVP + 5 µg purified Eta-1. By contrast, Eta-$1^{-/-}$ mice did not develop a detectable granulomatous response after challenge with PVP. However, coinjection of purified Eta-1 with PVP the partially restored the granulomatous response in Eta-$1^{-/-}$ mice (these experiments and Figure 1A).

Analysis of surface antigens expressed by cells within granulomas in the various mouse strains was done with monoclonal antibodies to Mac-1, B220, CD3, and BP-55 (a neutrophil marker). Histologic analysis of granulomas formed in Eta-$1^{-/-}$ mice

and in Eta-$1^{-/-}$ mice reconstituted with purified Eta-1 revealed a similar macrophage-dominant cellular infiltrate. About 85% of granulomatous cells in both cases were Mac-1^{+}, whereas 5 to 10% were $CD3^{+}$ T cells or $B220^{+}$ B cells. BP-55^{+} neutrophils, which were only a minor component (1 to 2%) of granulomas in these mice, increased 5- to 10-fold in granulomas firmed in IL-$10^{-/-}$ mice. Eta-$1^{-/-}$ mice also displayed defective granulomatous responses to injection of collagen and latex, consistent with reports that human T cells resident in sterile granulomas have high expression of Eta-1 (Nau *et al.* (1997) *Proc. Natl. Acad. Sci. U.S.A.* 94:6414; O'Regan *et al.* (1999) *J. Immunol.* 162:1024).

Lastly, the cytokine expression profiles were determined for cells from lymph nodes draining the site of granulomas in Eta-$1^{+/+}$ and Eta-$1^{-/-}$ mice. Briefly, PVP-dependent granulomas were formed as described above. After 5 days, mice were killed, and local lymph nodes were obtained for cytokine expression. Cytokine expression was measured 48 hours after incubation with PVP (2×10^6 cells per well). Restimulation of lymph nodes draining subcutaneous sites of PVP injection in Eta-$1^{-/-}$ mice and control mice with PVP revealed impaired IL-12 and interferon-γ (IFN-γ) responses. The IL-12 response was reduced by ~95%, and the IFN-γ response of Eta-$1^{-/-}$ mice was reduced by 90% in comparison to Eta-$1^{+/+}$ controls (Figure 1C).

Example 2: Eta-1/opn-dependent modulation of type-1 immunity and destructive type-1 autoimmune responses *in vivo* in herpes simplex virus-type 1 (HSV-1) infected control and Eta-1/opn-deficient mice

A second valuable *in vivo* animal model for studying type-1 immune responses involves inoculating mice (*e.g.*, corneal inoculation) with herpes simplex virus-1 ("HSV-1"). Inoculation with HSV-1 leads to delayed type immune responses in mice that can manifest as classical footpad swelling (Foster *et al* (1986) *Clin. Immunol. Immunopathol.* 40:313-325). Corneal HSV-1 infection can also lead to a destructive autoimmune inflammatory reaction, Herpes Simplex Keratitis (HSK), initiated by CD4 cells that recognize a viral peptide mimic of a murine corneal self-antigen (Zhao *et al.* (1998) *Science* 279:1344; Avery *et al.* (1995) *Nature* 376:431). This inflammatory response depends on the production of IL-12 and is inhibited by IL-10 (Streilein *et al.* (1997) *Immunol. Today* 18:443; Daheshia *et al.* (1997) *J. Immunol.* 159:1945).

Eta-$1^{-/-}$ mice were infected in the right eye with $4x10^6$ plaque-forming units (PFU) HSV-1 (KOS strain) and challenged five days later in the left footpad with $1x10^5$ PFU of UV-inactivated HSV-1 (KOS). Eta-$1^{-/-}$($opn^{-/-}$) mice infected by HSV-1 ($4x10^6$ PFU *via* the cornea) fail to develop a significant DTH response after footpad challenge with 10^5 pfu HSV-1, in contrast to the strong DTH response of Eta-$1^{+/+}$($opn^{+/+}$) controls (Figure 2A).

The numbers of T cells and proportions of T cell subsets in the thymus and peripheral lymphoid tissues of Eta-$1^{-/-}$ mice were similar to Eta-$1^{+/+}$ littermates. T and B cell subsets in Eta-$1^{-/-}$ and Eta-$1^{+/+}$ littermates were as follows: C57BL/6 × 129 Eta-$1^{+/+}$ spleen, 93.7×10^6 total cells (30.8% CD3, 19.8% CD4, 11% CD8, and 49.7% B220); C57BL/6 × 129 Eta-$1^{-/-}$ spleen, 82.6×10^6 cells (27.8% CD3, 18.8% CD4, 9.0% CD8, and 55.5% B220); C57BL/6 × 129 Eta-$1^{+/+}$ lymph node, 32.0×10^6 cells (82.4% CD3, 42.8% CD4, 34.2% CD8, and 12.8% B220); and C57BL/6 × 129 Eta-$1^{-/-}$ lymph node, 21.9×10^6 cells (82.8% CD3, 49.3% CD4, 28.4% CD8, and 11.2% B220). Moreover, T cells from Eta-$1^{-/-}$ and Eta-$1^{+/+}$ mice expressed levels of CD44 and CD62 that were not distinguishable. Although the T cell numbers and proportions were similar in Eta-$1^{-/-}$ and Eta-$1^{+/+}$ mice, the possibility existed that defective antiviral DTH response in Eta-$1^{-/-}$ mice might reflect a subtle alteration in lymphocyte or macrophage development. Accordingly, the effects of acute *in vivo* depletion of Eta-1 with a neutralizing antibody were tested in the Eta-$1^{-/-}$ mice. The neutralizing antisera LF-123 (Fisher *et al.* (1995) *Acta Orthop. Scand.* 66:61) or control normal rabbit serum were injected at 25 μg per dose per day, starting 2 days before injection. On day 0, mice were infected with HSV-1 (KOS) and rechallenged 5 days later. The right and left footpads of each mouse were measured 24 hours after rechallenge, and specific swelling (left versus right footpad) is shown in Figure 2B. Administration of antibody to Eta-1 (LF-123) immediately before and repeatedly after HSV-1 infection efficiently inhibited the DTH response upon rechallenge.

In a second experiment, Eta-$1^{-/-}$ and control mice (Eta-$1^{+/+}$) were subjected to ocular challenge with virus. As shown in Figure 2C, Eta-$1^{-/-}$ mice failed to develop significant HSK within 2 weeks after corneal inoculation with HSV-1 in contrast to the severe HSK developed within this period by control littermates (Eta-$1^{+/+}$) (*i.e.*, 65% of control Eta-$1^{+/+}$ mice developed HSK, Figure 2C). Similar results were

obtained when the experiment was repeated using BALB/cBγJ mice and CB-17 mice in addition to Eta-$1^{-/-}$ and Eta-$1^{+/+}$ mice, as demonstrated in Figure 2D. Furthermore, skewing of the cell numbers in Eta-1/opn knockout mice after challenge with HSV-1 was diminished compared to control mice in which the increase of $CD8^+$ cells is consistent with a Th-1 response.

Moreover, T-cells from Eta-$1^{-/-}$ mice are not impaired in their proliferative response to irradiated virus plus antigen presenting cells. The right superficial cervical draining lymph nodes of Eta-$1^{-/-}$ mice and Eta-$1^{+/+}$ littermate controls were harvested 15 days after infection of the right eye with 4×10^6 PFU of HSV-1 (KOS). Cells from these lymph nodes (2×10^6 cells per well) were incubated in the presence of 4×10^7 PFU of ultraviolet (UV)-inactivated HSV-1 (KOS). The proliferative response of lymph node cells from HSV-1-infected Eta-$1^{+/+}$ and Eta-$1^{-/-}$ mice measured by ^{3}H-thymidine incorporation at 72 h was 20.9×10^3 and 18.7×10^3 cpm, respectively. Furthermore, the absence of DTH does not reflect a general impairment of the immune system in these mice since clonal expansion followed by apoptosis after superantigen (SEB) injection were indistinguishable from wild-type mice. T cell expansion followed by apoptosis after superantigen (50 µg of staphylococcal enterotoxin B) intraperitoneal injection into Eta-$1^{-/-}$ and Eta-$1^{+/+}$ mice was indistinguishable at 3 days: +/+ $V_\beta 8^+$ CD4 cells (percentage of total spleen) increased from 3.6 to 5%; -/- $V_\beta 8^+$ CD4 cells increased from 3.2 to 5.5%; +/+ $V_\beta 6^+$ CD4 cells increased from 2.3 to 2.6%; -/- $V_\beta 6^+$ CD4 cells increased from 2.5 to 2.6%.

Although cells from the draining lymph nodes of virus-infected Eta-$1^{-/-}$ and Eta-$1^{+/+}$ mice respond equally well to HSV-1 according to [^{3}H]-thymidine incorporation after viral restimulation *in vitro*, they differed conspicuously according to their cytokine profiles. Briefly, cells were isolated and restimulated with HSV-1 (KOS) as described above. Supernatants were harvested 48h later and IL-10 and IL-12 p40 cytokine levels were measured by sandwich ELISA using OptIEA antibody sets (Pharmingen, La Jolla CA). IL-4 was measured after stimulation of draining lymph node cells by plate-bound anti-CD3. Cells from Eta-$1^{-/-}$ mice produced high levels of IL-10 and IL-4 but markedly reduced levels of IL-12, compared with Eta-$1^{+/+}$ controls (Figure 2E) and splenic macrophages from virus-infected Eta-$1^{+/+}$ but not Eta-$1^{-/-}$ mice continued to produce IL-12 ten days after infection. In contrast with the sterile granulomatous response, IFN-γ levels

were not reduced in Eta-$1^{-/-}$ mice after HSV-1 viral infection, consistent with an IL-12-independent pathway to IFN-γ production that may depend on virally induced IFN-α/β production (Oxenius *et al.* (1999) *J. Immunol.* 162-965; Cousens *et al.* (1999) *J. Exp. Med.* 189:1315). Moreover, expression of IL-2 by lymph node and spleen T lymphocytes from Eta-$1^{-/-}$ and Eta-$1^{+/+}$ littermates in response to immobilized antibody to CD3 was indistinguishable between the C57BL/6 × 129/SV Eta-$1^{-/-}$ and C57BL/6 × 129/SV Eta-$1^{+/+}$ mice. These cytokine profiles suggest that Eta-1/osteopontin expression normally may imprint the *in vivo* ratio of IL-12 and IL-10 cytokines that dictates a type-1 immunity.

Example 3: Treatment of HSV-1 infected mice with anti-Eta-1/osteopontin antibodies significantly downregulates type-1 immunity and destructive type-1 autoimmune responses

In a similar experiment to those described above, the levels of HSV-1-specific DTH reactions were measured 24 hours after footpad challenge in Cal20 mice that had been primed five days earlier by corneal inoculation of 4 x 10^4 to 4 x 10^7 plaque forming units ("pfu") of UV-inactivated HSV-1 and treated with anti-osteopontin antibody, LF-123 (Fisher *et al.* (1995) *Acta Orthop. Scand.* 66:61), or control serum every 48 hours. Mice treated only with control serum exhibited classical footpad swelling when footpads were measured 24 hours after challenge. By contrast, mice treated with LF-123 serum exhibited significantly diminished footpad swelling, indicating that neutralization of osteopontin significantly inhibited footpad swelling.

Table I: Specific footpad swelling in Cal20 mice in the presence of rabbit serum or anti-osteopontin antibody following HSV-1 inoculation (increasing pfus).

HSV-KOS	rabbit serum	LF-123
$4x10^4$	0.04 mm	0.07
$2x10^5$	0.1	0.05
$4x10^5$	0.42	0.03
$4x10^6$	0.57	0.13
$4x10^7$ (24 hr)	1.114 ..	0.6175

Moreover, as described above, HSV-1 infection of murine cornea leading to HSK results in corneal inflammation and destruction within 14 days after viral inoculation. Cal20 mice that were treated with anti-osteopontin antibody every 48 hours after challenge had reduced severity and incidence of HSK compared to mice injected with rabbit serum.

Table II: HSK in Cal20 mice in the presence of rabbit serum or anti-osteopontin antibody following HSV-1 inoculation

	Incidence*	Severity*
Cal20	77.77% 100%	2.11 3.1
Cal20 rabbit serum	50% 50%	2 2.25
Cal 20 LF-123	60% 60%	1.6 2

*day 11/day 14

Example 4: Eta-1/osteopontin-dependent modulation of protective immune responses following infection (*e.g., listeria monocytogenes* infection) *in vivo* in control and Eta-1/osteopontin-deficient mice

The murine response to *Listeria monocytogenes* is an experimental cornerstone of our understanding of the early events leading to type-1 immunity after microbial infection (Unanue (1997) *Immunol. Rev.* 158:11) and depends on early macrophage production of IL-12 and downstream expression of IFN-γ (Tripp *et al.* (1993) *Proc. Natl. Acad. Sci. U.S.A.* 90:3725; C. S. Tripp, Gately *et al.* (1994) *J. Immunol.* 152:1883; Tripp *et al.* (1995) *J. Immunol.* 155:3427). Accordingly, the ability of Eta-$1^{-/-}$ mice to mount a protective immune response after *Listeria* infection was investigated.

Listeria infection and cytokine production were as follows. Virulent *L. monocytogenes* (strain 1778, American Type Culture Collection (ATCC) designation 43251) was grown in trypticase soy broth, and 10^3 colony-forming units (CFU), a sublethal dose for this strain of *L. monocytogenes*, were injected intravenously into C57BL/6 (B6), B6-IL-$12^{-/-}$, B6-IL-$10^{-/-}$, B6 x 129-Eta-$1^{-/-}$, and B6 x 129-Eta-$1^{+/+}$ mice (Unanue (1997) *Immunol. Rev.* 158:11; Stordeur *et al.* (1995) *Mol. Immunol.* 32:233

(1995); Stordeur and Goldman (1998) *Int. Rev. Immunol.* 16:501). The titer of viable bacteria in the inoculum and in organ homogenates (*e.g.*, liver and spleen) was determined by plating 10-fold serial dilutions on trypticase soy agar plates. Plates were incubated at 37°C. and the numbers of CFU were counted after 24 hours. Eta-$1^{-/-}$ mice contained liver-associated *Listeria*-infected cysts that were apparent 4 (first experiment) and 5 (second experiment) days after infection (also seen in IL-$12^{-/-}$ mice). At 5 days (second experiment) *Listeria* infection was also evident in spleen homogenate of Eta-$1^{-/-}$ mice. These data demonstrate that Eta-$1^{-/-}$ mice were defective in their ability to clear *L. monocytogenes* after systemic infection, similar to the defect in IL-$12^{-/-}$ mice.

Restimulation of spleen cells from Eta-$1^{-/-}$ and Eta-$1^{+/+}$ mice with heat-killed bacteria revealed that cells from the former mice had reduced IFN-γ responses. Briefly, Spleen cells (4×10^6/ml) from four to five C57BL/6 × 129 Eta-$1^{+/+}$ or four to five C57BL/6 × 129 Eta-$1^{-/-}$ mice that had been intravenously inoculated 5 days earlier with 10^3 CFU were stimulated with heat-killed *L. monocytogenes* (2×10^8 CFU/ml) 96 hours before IFN-γ measurement by an OptEIA™ ELISA kit (PharMingen). 25.5 ± 6.5 ng/ml of IFN-γ were produced by spleen cells from Eta-$1^{+/+}$ mice in comparison with 3.2 ± 1.2 ng/ml of IFN-γ from Eta-$1^{-/-}$ mice.

The data presented in Examples 1-4 clearly demonstrate a role for Eta-1/osteomodulin in a variety of type-1 immune responses and demonstrate that type-1 immunity can be modulated by administration of purified Eta-1. The data presented in Examples 1-4 further indicate that Eta-1/osteopontin expression potentially effects type-1 immunity through regulation of the IL-12 and IL-10 cytokine ratio.

Example 5: Eta-1/osteopontin-dependent modulation of type-1 immunity cytokine profiles *in vitro*

This example further defines the role of Eta-1/opn in modulating immune effector cells, in particular, by demonstrating the ability of Eta-1/opn to modulate type-1 cytokine secretion *in vitro* (*e.g.*, in isolate peritoneal macrophages). Resident peritoneal macrophages were isolated from normal mice and treated with increasing amounts of purified Eta-1/opn. Briefly, peritoneal macrophages were obtained by peritoneal lavage (2x10 ml PBS) of C57BL/6 mice. Contaminating red cells were eliminated by hypotonic

lysis with ACK buffer. Cells were plated at 10^5/100 µl in 96-well plates and non-adherent cells were washed off after 2 hours. Adhered cells were then incubated for 48 hours with increasing concentrations of purified Eta-1/opn in serum-free medium and levels of IL-10 and IL-12 p75 in the supernatant were determined by ELISA (Figure 3A). Briefly, supernatant was withdrawn at the indicated time points for analysis of IL-10 or IL-12 p70 using commercial ELISA kits (R & D Systems). At the end of the incubation, the cells were tested for viability by propidium iodide incorporation (>98%) and their purity was confirmed by staining with fluorescence-conjugated anti-Mac1 antibody (>98%).

Treatment of cells with Eta-1/opn resulted in the secretion of as much as 400 pg/ml of IL-12 at 48 hours whereas IL-10 production was not detected (Figure 3A). Eta-1/opn-dependent induction of IL-12 secretion from macrophages was not due to contamination with endotoxin as Limulus ameboid lysate assay indicated that purified Eta-1/opn contained less than 1ng/g endotoxin. Moreover, quantities of endotoxin that escape detection in the limulus ameboid lysate assay do not contribute to biologic activity of Eta-1/opn because the IL-12 response of macrophages derived from C3H.HeJ mice (which are defective in endotoxin receptor-mediated signaling) was not impaired compared to other strains.

Next, resident peritoneal macrophages ($5x10^5$/ml) were treated with either 5 pmol/ml Eta-1/opn, 30 ng/ml LPS or 500 U/ml IL-4 and IL-12/IL-10 detected by ELISA at increasing times post-induction (Figure 3B). While LPS stimulation of these resident peritoneal macrophages induced both IL-12 (about 250 pg/ml) and IL-10 (about 100 pg/ml) and while IL-4 predominantly caused production of IL-10, Eta-1/opn selectively lead to secretion of IL-12. The failure of Eta-1/opn to induce IL-10 was somewhat surprising since other cytokines that activate macrophages (*e.g.*, TNFα, IL-1.IL-2.IL-3 and IL-6 all stimulate IL-10 secretion (Stordeur *et al.* (1995 *Mol. Immonol.* 32:233: Stordeur and Goldman (1998) *Int. Rev. Immunol.* 16:501), and lippopolysaccharide (LPS) stimulation of these resident peritoneal macrophages induced both IL-12 (~250 pg/ml) and IL-10 (~100 pg/ml).

Further analysis showed that Eta-1/opn actively suppressed IL-10 secretion by resident peritoneal macrophages stimulated with IL-4 (Figure 3C). Briefly, macrophages were activated with IL-4 (500 U/ml x 1 hour) before addition of Eta-1/opn (5

pmol/ml) for an additional 48 h before measurement of IL-12 and IL-10 by ELISA. In some groups, anti-IL-12 (R & D Systems, Minneapolis, MN) was added at a final concentration of 2 μg/ml. As shown in Figure 3C, IL-4-dependent induction of macrophage IL-10 was inhibited by the addition of Eta-1/opn, but this effect was not altered by anti-IL-12 neutralizing antibody, suggesting a direct mode of action.

Moreover, Eta-1 actively suppressed the LPS dependent IL-10 response of resident peritoneal macrophages (Figure 3D). Briefly, macrophages were activated with LPS (30 ng/ml) for 1 hour before addition of Eta-1 (5 nM) for an additional 48 hours and consecutive measurement of IL-12 and IL-10 by ELISA. Assays were performed in quadruplets, and each point represents the mean and standard error (error bars) of two independent experiments.

Examples 6-12 define the functional domains of Eta-1/osteopontin and map various Eta-1/osteopontin-dependent activities to their respective domains. These Examples also define various bioactive fragments of Eta-1 for modulating immune effector cell activation (*e.g.*, cell motility, spreading, cytokine and metalloproteinase secretion). These examples also describe the phosphorylation dependence of various Eta-1/osteopontin-dependent activities.

Additional Materials and Methods for Examples 6-12

Cell lines: A31 is an integrin $\alpha_v\beta_3$, CD44 murine embryonic fibroblast clone derived from Balb 3T3 cells (CCL-163, ATCC). A31 cells transfected with CD44 (A31.C1) or A31 mock-transfectants were generated as described (Weber *et al.* (1996) *Science* 26:271:509-512). MH-S is a macrophage cell line that was derived by SV40 transformation from an adherent cell enriched population of alveolar macrophages (CRL-2019, ATCC). MT-2/1 is a thymus-derived macrophage from a Balb/c mouse that was immortalized by infection with retroviral vector. It expresses CD44 and integrin $\alpha_v\beta_3$.

Eta-1/opn purification and cleavage: To generate recombinant Eta-1/opn, GST-Eta-1/opn fusion protein was expressed in *E. coli*, digested with factor Xa, and purified by affinity chromatography as described (3 refs). For phosphorylated recombinant Eta-1/opn, GST-Eta-1/opn (5 mg) was incubated with10 μg of Golgi kinase

for 2 h before passage through a GSH-Sepharose column and elution from GSH-Beads with 100 U of factor Xa. The eluate was applied to a chromatofocusing column and eluted from the resin with polybuffer 74 as described above. The major peak eluted at pH 4.6 and phospho-amino acid analysis of the recovered protein revealed a phosphoserine content of 16 mol of phosphate/mol protein and 0.8 mols of phosphothreonine/mol protein. Native Eta-1/opn were prepared as described above. Thrombin cleaves Eta-1/opn into two fragments following the arginine in the sequence VVYGLR in Eta-1/opn (*e.g.*, amino acid residues 162-168 of SEQ ID NO:2), an N-terminal fragment ("Eta-1/opn NT) containing the RGD motif and a C-terminal fragment ("Eta-1/opn CT"). Thrombin cleavage and phosphorylation of either the dephosphorylated native protein or recombinant Eta-1/opn was accomplished by human thrombin (Sigma Chemicals), Golgi kinases or purified casein kinase II or casein kinase I.

Chemotaxis: Directed migration of cells was determined in multi-well chemotaxis chambers as described (Weber *et al.* (1996) *Science* 26:271:509-512). Briefly, two-well culture plates (Transwell) with polycarbonate filters (pore size 8-12 μm) separating top and bottom wells were coated with 5μg fibronectin. 2 X 10^5 cells were added to the upper chamber and incubated at 37° C in the presence or absence of Eta-1/opn in the lower chamber. After 4 h, the filters were removed, fixed in methanol, stained with hematoxylin and eosin and cells that had migrated to various areas of the lower surface were counted microscopically. Controls for chemokinesis included 200 ng of the appropriate form of osteopontin in the top well. All assays were done in triplicates and are reported as mean ± standard deviation.

Haptotaxis: Haptotaxis of monocytic cell lines to Eta-1/opn or fragments of Eta-1/opn was assayed using a Boyden chamber. The lower surface or both sides of polycarbonate filters with 8 μm pore size were coated with the indicated amounts of Eta-1/opn. 2 X 10^5 cells were added to the upper chamber, and incubated at 37° C in the absence of any factors in the lower chamber. After 4 h, the filters were removed, fixed in methanol and stained with hematoxylin and eosin. Cells that had migrated to the lower surface were counted under a microscope. All assays were done in triplicates and are reported as mean ± standard deviation.

Cell attachment and spreading: 24-well plates were coated over night at 4°C with 10 μg/ml of the indicated ligand then blocked for 1 h at room temperature with 1-10 mg/ml BSA in PBS. To preserve the integrity of adhesion receptors, MH-S monocytic cells were harvested from subconfluent cultures by non-enzymatic cell dissociation solution (Sigma, St Louis MO). Cells were washed twice with PBS and resuspended at a concentration of 1 X 10^5 cell/ml of sterile Ca^{2+} and Mg^{2+}-free PBS supplemented with 0.1% BSA and 1 mM sodium pyruvate. 5 x 10^3 to 5 x 10^4 cells were incubated in each well and, after 1 h at 37°C, the wells were washed 3 times with 0.5 ml PBS to remove non-adherent cells, fixed in 10% buffered formalin, 1% paraformaldehyde, or 1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at room temperature for ~1 hour then stained with toluidene blue and hematoxylin. The total number of attached or spread cells in each well were counted microscopically using a Nikon Eclipse microscope equipped with a Sony digital Camera. Total number of attached or spread cells were quantitated using Optima 5.2 image analysis system. Each experiment was done in triplicates and is reported as mean ± SEM. To minimize variability inherent to cell attachment studies, cells were scored as attached only when a defined nucleus was observed, accompanied by a transition from round to cuboidal cell morphology. Round cells that are loosely attached with no defined nucleus were scored as non-attached. These cells can be removed with repeated washes. The viability of the cells was measured before and after the termination of the experiments and only data from experiments with greater than 95 % cell viability were used. Further, under the conditions used in these experiments, cell attachment was temperature dependent, inhibitable by trypsin treatment and not affected by inhibitors of protein synthesis or secretion. Cell spreading was determined by membrane contour analysis and was scored according to increase in cell volume/surface area. In some experiments, cell spreading was also assessed by the formation of stress fibers. Each experiment was performed in quadruplicate wells and repeated 3 times.

Example 6: Effects of Various Eta-1/opn Domains on Cellular Chemotaxis

This example describes the domain-specific effects of Eta-1/osteopontin on chemotaxis of immune effector cells (*e.g.*, monocytes).

Stable CD44 transfectants of $\alpha_v\beta_3$ fibroblasts (Weber *et al.* (1996) *Science* 26:271:509-512) were used to examine the interaction of CD44 with osteopontin. Chemotactic activity of Eta-1/opn or Eta-1/opn fragments was tested in a modified Boyden chamber (Weber *et al.* (1996) *Science* 26:271:509-512). Purified natural osteopontin exerted chemotactic activity for the MH-S monocyte cell line. Moreover, the C-terminal thrombin cleavage product but not the N-terminal cleavage fragment mediated chemotaxis of A31.C1 (CD44 stably transfected cells), but not mock-transfected A31.MLV cells, confirming the dependence of cell migration on the expression of CD44. The C-terminal fragment of Eta-1/opn also induced chemotaxis of macrophage cell line MH-S as efficiently as intact Eta-1/opn, whereas the N-terminal 30 kDa Eta-1/opn fragment was inactive.

Table III: Chemotactic Response of MH-S Cells to Eta-1/opn

	LOWER CHAMBER			
UPPER CHAMBER	PBS	Eta-1/opn	Eta-1/opn CT	Eta-1/opn NT
PBS	56±10	312±56*	478±98*	71±21
Eta-1/opn	34±7	168±24	305±50	36±19
Eta-1/opn CT	9±4	88±24	220±38	26±5
Eta-1/opn NT	63±11	287±60	409±55	14±5

* $P<0.01$

Moreover, equimolar mixtures of both fragments displayed activity similar to that of the 28 kDa C-terminal fragment alone. These results, taken together, indicate that the chemotactic domain of Eta-1/opn resides in the 28 kDa C-terminal part of the molecule. This C-terminal fragment-mediated activity could further be inhibited by Eta-1/opn fragments and various modulators of receptor interaction.

Table IV: Inhibition of Monocyte Chemotaxis

	MI Eta-1/opn	MI Eta-1/opn CT
Control	13.3 ± 1.9	9.6 ± 1.9
+ GRGDS† (1mM)	10.6 ± 1.3	7.7 ± 1.6
+ anti CD44 (0.1 μg)	7.8 ± 0.7**	4.6 ± 0.7*
+ anti β3 (0.1 μg)	9.8 ± 0.6	12.1 ± 2.1
+ Eta-1/opn NT	12.3 ± 2.0	9.6 ± 1.9
+ Eta-1/opn CT	6.3 ± 0.7*	3.7 ± 1.6*
+Eta-1/opn	4.4 ± 0.6*	2.6 ± 0.2*

* P<0.01 ** P<0.05 † (SEQ ID NO:11)

Previous investigations of stable transfectants of A31 cells showed that the interaction of Eta-1/opn with CD44 depended on expression of CD44 splice variants 3-6 (Weber *et al.* (1996) *Science* 26:271:509-512), which characterize activated lymphocytes (Arch *et al.* (1992) *Science* 257:682-685). More recent studies indicate that A31 cells transfected with the standard form of CD44 (lacking variant exons) do not bind Eta-1/opn.

Example 9: Effects of Various Eta-1/opn Domains of Hapotaxis

This example describes the domain-specific effects of Eta-1/osteopontin on haptotaxis of immune effector cells (*e.g.*, monocytes).

Cells can move up a gradient of immobilized ligand, a process referred to as haptotaxis. This cell crawling may occur on vessel walls or in the interstitium. Therefore, the contribution to cell motility of interactions between immobilized Eta-1/opn, Eta-1/opn fragments, and integrin receptors was assessed as follows. The ability of the immobilized ligand to induce monocyte haptotaxis was judged by cell migration through poly-carbonate filters. Eta-1/opn induced monocyte migration that was mainly directional (*i.e.*, the cells responded to a positive gradient of bound Eta-1/opn), and thus haptotactic and was inhibited by GRGDS (SEQ ID NO:11) and antibody to the β_3 chain

of integrins but not by antibody to CD44. Data are expressed as migratory index (cells migrating in response to Eta-1/opn/cells migration in response to buffer). Values are expressed as mean ± SEM.

Table V: Hapotactic Response of Monocytes to Eta-1/opn

	Eta-1/opn Bound to Upper Side			
Eta-1/opn Bound to Lower Side	0 pmol	30 pmol	90 pmol	150 pmol
0 μg	1.0 ± 0.15	1.5 ± 0.2	1.8 ± 0.1	0.6 ± 0.2
1	5.2 ± 0.35	1.5 ± 0.3		
3	7.8 ± 0.4		2.1 ± 0.3	
5	9.3 ± 0.8			2 ± 0.1

Table VI: Inhibition of Monocyte Hapotaxis

	MI Eta-1/opn	MI Eta-1/opn-NT
control	9.8 ± 0.9	6.1 ± 0.7
+ GRGDS† (1mM)	3.6 ± 1*	2.1 ± 0.2*
+ anti CD44 (0.1 μg)	7.8 ± 0.7	5.5 ± 0.3
+ anti integrin β3	4.8 ± 0.6*	1.6 ± 0.1*

* $P<0.01$ † (SEQ ID NO:11)

Table VII: Effects of Eta-1/opn Phosphorylation on Hapotaxis and Chemotaxis

	Phosphorylated Eta-1/opn		Unphosphorylated Eta-1/opn	
	Hapotactic Index	Chemotactic Index	Hapotactic Index	Chemotactic Index
Control	1 ± 0.1	1.3 ± 0.3	1 ± 0.3	1 ± 0.2
Eta-1/opn	9.8 ± 0.9*	13.3 ± 1.9*	3.6 ± 0.6**	11.4 ± 1.8*
Eta-1/opn NT	1.8 ± 0.7	0.9 ± 0.1	0.6 ± 0.2	0.9 ± 0.2
Eta-1/opn CT	1.6 ± 0.5	9.6 ± 1.9*	1.3 ± 0.6	9.6 ± 1.9*
NT10k	4.2 ± 1.1**	1.1 ± 0.2	1.8 ± 0.9	1.1 ± 0.1
rEta-1/opn	-	-	1.5 ± 0.1	10.5 ± 2.2*
rEta-1/opn (GK)	12.6 ± 2.1*	10.4 ± 1.6*	-	-
rEta-1/opn (CKII)	8 ± 1.8*	10.3 ± 1.6*	-	-
rEta-1/opn (CKI)	10 ± 1.9*	9.9 ± 2.3*	-	-
rEta-1/opn (PKG)	0.8 ± 0.4	8.7 ± 2.0*	-	-

Example 8: Effects of Eta-1/opn and Various Eta-1/opn Domains on Cellular Spreading

This example describes the domain-specific and phosphorylation-dependent effects of Eta-1/osteopontin on the spreading of immune effector cells (*e.g.*, the spreading of monocytes).

Macrophage spreading on extracellular matrix proteins depends, in part, on engagement of their integrin receptors. MH-S cells attached and spread on immobilized phosphorylated Eta-1/opn whereas MH-S cells plated on unphosphorylated Eta-1/opn did not spread (as determined microscopically). Spreading of the MH-S macrophage cell line on immobilized native Eta-1/opn is mediated by the RGD-containing N-terminal thrombin cleavage fragment but not by the C-terminal fragment and is reversed by addition of soluble GRGDS (SEQ ID NO:11) but not control GRGES (SEQ ID NO:12) peptide (Figure 4A).

Moreover, phosphorylation of recombinant Eta-1/opn is required for this activity. rEta-1/opn was phosphorylated with the indicated kinases as previously described (Ashkar, 1993, 1993b, 1995, Salih,1997). rEta-1/opn (GK), recombinant Eta-1/opn phosphorylated with golgi kinases isolated for mouse calvarial cells (14 mol of

phosphate/mol protein); rEta-1/opn (CKII) rEta-1/opn phosphorylated with casein kinase II (9 mol phosphate/mol protein) rEta-1/opn (CKI) phosphorylated with casein kinase I (11 mol phosphate/mol protein) rEta-1/opn (PKG) recombinant Eta-1/opn phosphorylated with cGMP dependent protein kinase (3 mol phosphate/mol protein). Since none of the sites are phosphorylated 100% the mol phophate/mol protein does not reflect the total number of sites phcsphorylated.

Table VIII: Spreading Indices Indicate the Effect of Phosphorylation of Recombinant Eta-1/opn (rEta-1/opn) and Dephosphorylation of Native Eta-1/opn

	Phosphorylated Eta-1/opn	Unphosphorylated Eta-1/opn
Control	1 ± 0.3	1 ± 0.1
Eta-1/opn	10 ± 2.1*	2 ± 0.7
Eta-1/opn NT	11 ± 1.6*	11 ± 1.5
Eta-1/opn CT	2 ± 0.7	2 ± 0.8
NT10k	5 ± 2.4**	7 ± 0.5
REta-1/opn	-	2.4 ± 0.3
rEta-1/opn (GK)	11 ± 1.7*	-
rOEta-1/opn (CKII)	12 ± 3.1*	-
rEta-1/opn (CKII)	8 ± 2.6*	-
Eta-1/opn(PKG)	1 ± 0.1	-

Cleavage of Eta-1/opn with thrombin exposes the RGD motif and may enhance its cell attachment properties (Senger *et al.* (1995)). Mutagenesis of the RGD sequence substantially reduced attachment of melanoma cells (Smith *et al.* (1998)), tumor cells, and gingival fibroblasts (Xuan *et al.* (1995)) demonstrating the necessity of this motif.

While the RGD sequence is necessary for integrin binding, it is not specific for a particular integrin receptor. Eta-1/opn may be secreted in nonphosphorylated (Kubota *et al.* (1989) *Biochem. Biophys. Res. Comm.* 162:1453-1459; Chambers *et al.* (1992) *Anticancer Res.* 12:43-47; Barak-Shalom *et al.* (1995) *Comp. Biochem. Physiol.* 111:49-59; and Chang and Prince (1993) *Cancer Res.* 53:2217-2220) and phosphorylated forms that contain up to 28 phosphate residues

(Sorensen and Peterson (1994) *Biochem. Biophys. Res Comm.* 198:200-205; Salih *et al.* (1995) *Ann. NY Acad. Sci.* 760:357-360; Salih *et al.* (1997) *J. Biol. Chem.* 272:13966-13973. Phosphorylation is functionally important because it may determine whether Eta-1/opn associates with the cell surface or with the extracellular matrix and it may be essential for integrin-mediated cell adhesion. Therefore, phosphorylation of the molecule may provide selectivity of integrin binding. Phosphorylation has to occur at specific sites because Golgi kinases and casein kinases I or II can activate Eta-1/opn whereas protein kinases A or G phosphorylate the recombinant molecule but do not confer integrin binding.

In a second experiment, MH-S cells attached to, but did not spread on phosphorylated and unphosphorylated PNGRGDSLAYGLR (SEQ ID NO:13) synthetic peptides. In an attempts to define an N-terminal peptide capable of support attachment and spreading, partial tryptic, chemotryptic and Asp-N endopeptidase digestion of Eta-1/opn was performed. None of these, however, resulted in the isolation of an active peptide. A 10-kD fragment isolated from a Lys-C digest was found to be active. NK10 has the NH_2-terminal sequence QETLPSN (SEQ ID NO:14) and is predicted to terminate at the thrombin cleavage site. This 10-kD fragment also contains ~5 mol of phosphate per 1 mol of peptide at seven potential phosphorylation sites. NK10 was capable of mediating the spreading of macrophages at approximately 40 % (mol/mol) the activity of the larger N-terminal thrombin fragment (Figure 4B). Upon dephosphorylation of this peptide spreading activity is lost, but can be regained by rephosphorylation with Golgi kinases. Earlier studies which showed that RGD-containing peptides can confer function may have induced non-specific effects through multiple integrin receptors. These data demonstrate that the RGD motif is necessary but not sufficient to mediate specific Eta-1/opn activity, phosphorylation in defined sites is also needed.

Example 9: Eta-1/osteopontin-dependent modulation of type-1 immunity cytokines *via* distinct receptors on immune effector cells (*e.g.*, macrophages)

As shown in Examples 6-8, Eta-1/opn interaction with macrophages is mediated through two distinct functional receptors. Engagement of CD44 mediates

chemotactic migration and interaction with $\alpha_v\beta_3$ integrin causes haptotaxis, adhesion and spreading.

To determine whether distinct macrophage receptors were responsible for the type-1 cytokine production by Eta-1/opn stimulated macrophages, fragments from a Lys-C digest of Eta-1/opn were analyzed for the ability to stimulate IL-12 secretion. The 10 kDa NK10 proteolytic fragment from the N-terminal portion of Eta-1/opn containing the integrin binding site was found to be sufficient to induce macrophage IL-12 expression (Figure 5A).

In contrast to IL-12 induction, inhibition of IL-10 depends on engagement of the CD44 receptor: Figure 5B shows that Eta-1/opn-dependent inhibition of IL-4-induced production of IL-10 was reversed by anti-CD44 (KM81, purified from ATCC hybridoma TIB 241, described in Mayake *et al.* (1990) *J. Exp. Med.* 171:477-488) but not anti-integrin β_3 antibody (Pharmingen, described in Schultz and Armant (1995) *J. Biol. Chem.* 270:11522). Moreover, macrophages from $CD44^{-/-}$ mice are resistant to Eta-1/opn inhibition of the IL-10 response. Figure 5C shows that secretion of IL-12 in response to Eta-1/opn was not impaired in macrophages from mice that are deficient in the CD44 gene and cells from C3H.HeJ mice (which do not respond to endotoxin) displayed the same levels of induction as control mice. Conversely, while the inhibition of IL-10 secretion was not affected in C3H.HeJ mice or in C57Bl/6 mice, it was abrogated in $CD44^{-/-}$ mice.

Example 10: Phosphorylation of Eta-1/osteopontin is necessary for engagement of integrin receptors on macrophages leading to IL-12 production but not for ligation of CD44 leading to IL-10 inhibition

Eta-1/opn is secreted in nonphosphorylated and phosphorylated forms (ref.). Phosphorylation may allow Eta-1/opn to associate with the cell surface rather than the extracellular matrix (refs) through a contribution to integrin binding. In contrast, serine phosphorylation of recombinant Eta-1/opn is not required for CD44-dependent interactions leading to chemotactic migration (ref.). To determine whether phosphorylation of Eta-1/opn might affect its ability to regulate cytokine expression, phosphorylated and dephosphorylated Eta-1/opn were tested for their ability to affect IL-12/IL-10 secretion.

Figure 6A demonstrates that dephosphorylated purified naturally-produced Eta-1/opn abolished IL-12 stimulatory activity whereas phosphorylation of recombinant Eta-1/opn at specific sites restores activity. Secretion of IL-12 was measured by ELISA after culture of resident peritoneal macrophages with 6 pmol/ml of dephosphorylated natural Eta-1/opn (dpEta-1/osteopontin), recombinant Eta-1/opn (rEta-1/osteopontin) or recombinant phosphorylated Eta-1/opn (rEta-1/osteopontin~P) for 6 h in defined medium at 37°C. Dephosphorylated native Eta-1/opn and recombinant (unphosphorylated) Eta-1/opn does not induce IL-12 production but retains inhibitory activity for IL-10. Recombinant Eta-1/opn phosphorylation with Golgi kinases (rEta-1/osteopontin~P) conferred IL-12 inducing activity while similar levels of phosphorylation by PKA and PKC did not restore this activity.

Although recombinant Eta-1/opn lacking phosphate groups cannot induce IL-12, this molecule retains inhibitory activity for the macrophage IL-10 response (Figure 6B). Dephosphorylation of native Eta-1/opn resulted in loss of IL-12 inducing activity, while phosphorylation of (inactive) recombinant Eta-1/opn restored this function. There is abundant evidence that phosphorylation can regulate the biological activity of intracellular enzymes and their substrates; these results indicate that serine phosphorylation can also provide molecular information that regulates the biological activity of a secreted protein.

Figure 7 depicts cytokine profiles for macrophages after engagement by phosphorylated versus unphosphorylated Eta-1/opn, as well as by Eta-1/opn fragments. TNFα, TGFβ, and the type-1 cytokine IL-12 as well as the type-1 cytokine IL-10 were determined by commercial ELISA kits. (For induction of TGFβ cells were cultured on the indicated ligand for 6 h in defined media at 37° C in a humidified atmosphere.) Cytokine secretion data is presented as fold induction over resting values. Ligation of integrin receptors (*e.g.*, by native Eta-1/opn, recombinant phosphorylated Eta-1/opn, N-terminal fragment or NK10) on macrophages caused predominantly secretion of IL-12, TNFα, TGFβ but not IL-10 or IL-1α (*e.g.*, a type-1 cytokine profile).

Example 12: Signal Transduction Pathways Associated with Eta-1/opn-Mediated Functions Including Chemotaxis, Hapotaxis and Cell Spreading

This example demonstrates that distinct cellular signaling mechanisms are activated by association of the two key functional domains of Eta-1/osteopontin with their respective receptors on macropages.

Three biological response phenotypes have been observed in association with ligation of CD44 and integrin $\alpha_v\beta_3$: chemotaxis, cell crawling, and activation (after spreading on substrate). Accordingly, it was tested whether these functions were distinguishable on the level of intracellular signal transduction. Signal transduction mechanisms were initially examined through the use of specific chemical inhibitors at the following final concentrations: 50 mM for cycloheximide, PKA inhibitors H89 at lmM and, H7 at 20 uM, Inhibitors of PI pathway Wortmannin at 10 nM, tyrosine kinase inhibitors genistein at 25 uM for, PKC inhibitor chelerythrine at 20 uM, Casein Kinase II inhibitor quercetin at 6 mM. In all experiments using these compounds cells were preincubated for 0.5 hours with the inhibitors before start of the experiment. Cell viability was determined by trypan blue exclusion on cell samples before and after the termination of the experiments. Cell viability in all reported experiments was > 95 %. Microfilament disruption was carried out by preincubation of the cell cultures for one hour in 50 uM cytochalasin D. Microtubule dissociation was carried out by pre-incubation of the cultures for 6 hours in 1 μM colchicine. All compounds were suspended in either DMSO or absolute ethanol and were added to the culture media at 1:1000 dilution. Controls were carried out with the corresponding vehicle. In separate experiments in which PKC and PKA were chemically activated, 50 ng/ml phorbol 12-myristate 13-acetate and 10^5 M of forskolin were used respectively. In these experiments treatments were for 2 hours.

In order to delineate the mechanisms mediating differential activities of Eta-1/opn following ligation of its two groups of receptors, the effects of protein kinase inhibitors, cytoskeletal disrupting agents, and toxins were tested on the macrophage responses. Eta-1/opn mediated chemotaxis is diminished by the G-protein inhibitor pertussis toxin but not by inhibitors of protein kinase C or A, in contrast haptotaxis is not affected by pertussis toxin or protein kinase A inhibitors but is inhibited by the protein kinase C inhibitor chelerythrine.

Table IX: Inhibition of Monocyte Hapotaxis

	MI Eta-1/opn	MI Eta-1/opn
Control	9.8 ± 0.9	6.1 ± 0.7
+ Wortmanin (10nM)	10.1 ± 2.2	8.8 ± 1.3
+ Chelerythrine (20μM)	3.3 ± 0.2*	1.3 ± 0.1*
+ Genistein (25μM)	3.2 ± 0.5**	2.0 ± 0.3
+ PT	10.6 ± 1.1*	9.4 ± 1.5*
+ H 7 (20μM)	10.8 ± 1.7	6.9 ± 2.0
+ Cytocholasin D (1μM)	1.1 ± 0.2*	2.7 ± 0.1*

* P<0.01

Table X: Inhibition of Monocyte Chemotaxis

	MI Eta-1/opn	MI Eta-1/opn CT
control	13.3 ± 1.9	9.6 ± 1.9
+ Wortmanin (10nM)	12.6 ± 2.5	10.5 ± 1.6
+ Chelerythrine (20μM)	3.1 ± 0.5*	1.9 ± 0.8*
+ Genistein (25μM)	6.6 ± 1.1**	4.3 ± 0.7
+ PT	2.3 ± 0.21*	1.6 ± 0.3*
+ H 7 (20μM)	14.1 ± 2.7	8.7 ± 1.2
+ Cytocholasin D (1μM)	1.8 ± 0.9*	2.2 ± 0.3*

*P<0.01 **P<0.05

Ligation of integrin receptors by Eta-1/opn may lead to dephosphorylation of Src in chicken osteoblasts and recombinant Eta-1/opn may phosphorylate paxillin, tensin, and pl25 focal adhesion kinase in ras-transformed NIH3T3 cells. G proteins are linked to ligation of CD44 by Eta-1/osteopontin. Ligation of integrin $\alpha_v\beta_3$ initially leads to activation of PKC (see *e.g.*, Figures 8 and 9). After cell spreading, the cytoskeleton rearranges and a second integrin-associated signal transduction component, phosphatidylinositol 3-kinase, is activated (Figure 4B). Spreading of macrophages on Eta-1/opn is inhibited by chelerythrine and by inhibitors of the phosphatidylinositol pathway consistent with earlier reports that engagement of integrin $\alpha_v\beta_3$ on osteoclasts by Eta-1/opn leads to activation of phosphatidylinositol 3-

hydroxyl kinase . Direct measurement of phosphorylation of phosphatidylinositol 3-kinase is in accord with the inhibitor-based observations. The selective inhibition of macrophage responses argues against a toxic effect of these inhibitors.

The data in Example 12 demonstrate that CD44-dependent chemotaxis is associated with a signal transduction pathway that involves G-protein, while integrin-dependent haptotaxis is mediated by a pathway involving protein kinase C. Once a cell has spread, phosphatidylinositol signaling is integrated as a second component into integrin-dependent signaling. Distinct macrophage phenotypes induced by Eta-1/opn can be separated on the level of signal transduction using G-protein, protein kinase C, and phosphatidylinositol 3-kinase as biochemical markers.

Example 11: Domain-Specificity and Phosphorylation-Dependence of Induction of Metalloprotease Secretion by Eta-1/osteopontin

Because cell spreading is often associated with cellular activation, an investigation was made into whether the interaction between phosphorylated Eta-1/opn and macrophages leads to additional signs of macrophage activation including secretion of metalloproteinases and cytokines.

For metalloprotease secretion assays, MH-S cells were stimulated for 6 hours with either phosphorylated or unphosphorylated Eta-1/opn at a concentration of 10 µg/ml in serum-free defined medium. In order to visualize the secreted metalloproteases, gelatin zymograms were performed. Briefly, cell culture supernatant was collected after 6 hours of culture, concentrated 5 times and resuspended in 200 µl zymogram buffer (40 mM Tris, pH 7.5) before addition to Laemmli sample buffer and electrophoresis in 10% polyacrylamide gels impregnated with 1mg/ml gelatin. Following electrophoresis, gels were incubated for 30 min at 37°C in 50 ml of 50 mM Tris-HCl buffer, pH 8.0, containing 2% Triton-X 100 and 10 mM $CaCl_2$ to remove the SDS, followed by incubation for 18 h in 50 mM Tris-HCl buffer, pH 8.0, containing 5 mM $CaCl_2$. After staining the gels with Coomassie Brilliant Blue, gelatin and casein degrading enzymes were identified as clear bands against a dark blue background.

MMP-9 and MMP-2 were both visible in the samples stimulated with natural Eta-1/opn or with phosphorylated rEta-1/opn. Control MH-S cells were incubated with serum-free defined medium. MMP9 but not MMP2 was stimulated by the N-terminal

fragment of osteopontin, while the C-terminal fragment of Eta-1/opn had little or no stimulatory activity. MMP-9 induction could be inhibited by GRGDS (SEQ ID NO:11) but not GRGES (SEQ ID NO:12). Dephosphorylation of Eta-1/opn with acid phosphatase abolished the stimulatory activity of Eta-1/opn. Similarly, rEta-1/opn had no stimulatory activity. The results demonstrate that only phosphorylated Eta-1/opn had a stimulatory effect the gelatinolytic activity secreted by MH-S cells.

Definition of the functional domains of Eta-1/opn in the examples described above represents an important step in understanding this process and is critical for the rational development of Eta-1/opn analogs that antagonize or mimic discrete biological activities of the parent molecule. Examples 13-14 describe the generation and testing of such analogs.

Example 13: Generation of Biosynthetic Immunomodulatory Molecules That Stimulate IL-12

A first generation osteopontin-derived biosynthetic molecule was engineered based on the isolation of a domain of osteopontin sufficient to impart IL-12 stimulatory activity when isolated from the naturally-occurring polypeptide. Figure 10 depicts the amino acid and encoding nucleic acid sequence of such a molecule, termed immunomodulin-1, based on its ability to modulate immune responses. In particular, the biosynthetic immunomodulin-1 molecule depicted in Figure 10 has the ability to bias an immune response from a type-2 response to a type-1 response.

Example 14: Generation of Biosynthetic Immunomodulatory Molecules That Inhibit IL-10

A first generation osteopontin-derived biosynthetic molecule was engineered based on the isolation of a domain of osteopontin sufficient to impart IL-10 inhibitory activity when isolated from the naturally-occurring polypeptide. Figure 11 schematically depicts the structure of such a molecule, termed immunomodulin-2, based on its ability to modulate immune responses. In particular, the biosynthetic immunomodulin-2 molecule depicted in Figure 11 has the ability to bias an immune response from a type-2 response to a type-1 response.

Example 15: Testing of Immunomodulin-1 and Immunomodulin-2 *in vitro* and *in vivo*

Immunomodulin-1 and Immunomodulin-2 were tested for their ability to stimulate and/or inhibit cytokine secretion, in particular for their ability to stimulate and/or inhibit secretion of IL-12 and/or IL-10. As shown in Figure 12, Immunomodulin-1 is capable of stimulating IL-12 secretion by macrophages to levels greater than those induced by LPS. Moreover, as demonstrated in Figure 13, IL-10 is capable of inhbiting IL-4 induced IL-10 secretion by macrophages. The data demonstrate that Immunomodulin-1 and –2 are capable of biasing an immune response towards a type-1 response *in vitro*.

In order to test the ability of Immunomodulin-2 to bias an immune response *in vivo* from a type-2 to a type-1 response, C57blk mice were sensitized with a single intraperitoneal injection of 0.1 μg/ml poke weed adsorbed to 2 mg aluminum hydroxide. Animals were challenged at 7 or 14 days following sensitization with either aerosol poke weed (1μg/ml in an atomizer) or *via* subcutaneous injection of 0.05 μg/ml poke weed in phosphate-buffered saline (PBS). Plasma levels of IgE were determined by ELISA using antibodies to mouse IgE. Immunomodulin-2 was injected intraperitoneally (100 μl at a concentration of 10 μg/ml in PBS). Plasma concentrations of IgE were determined 3 and 14 days after injection (Figure 14).

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

What is claimed:

1. A method of modulating a type-1 immune response in a subject comprising administering to said subject an Eta-1/osteopontin modulator such that the type-1 immune response is modulated.

2. The method of claim 1, wherein the Eta-1/osteopontin modulator stimulates Eta-1/osteopontin activity and the type-1 immune response is potentiated.

3. The method of claim 1, wherein the Eta-1/osteopontin modulator inhibits Eta-1/osteopontin activity and the type-1 immune response is downregulated.

4. The method of claim 1, wherein the subject is a human subject.

5. The method of claim 1, wherein the Eta-1/osteopontin modulator is administered in a therapeutically effective amount.

6. The method of claim 1, further comprising monitoring the type-1 response in said subject.

7. The method of claim 6, wherein monitoring the type-1 response comprises determining the level of a detectable indicator of the type-1 response.

8. The method of claim 7, wherein monitoring the type-1 response further comprises comparing the level of the detectable indicator to a control.

9. A method of potentiating a type-1 immune response in a patient comprising:

(a) selecting a patient suffering from a disorder that would benefit from a potentiated type-1 immune response: and

(b) administering to said patient an Eta-1/osteopontin stimulatory modulator such that the type-1 immune response is potentiated.

10. The method of claim 9, wherein the disorder is selected from the group consisting of burn-associated sepsis, bacterial infection, viral infection, parasitic infection, mycoplasma infection, fungal infection, cancer, immunodeficiency disorders, AIDS, bone marrow transplant-related immunodeficiency, chemotherapy-related immunodeficiency and allergy.

11. A method of downregulating a type-1 immune response in a patient comprising:

(a) selecting a patient suffering from a disorder that would benefit from a downregulated type-1 immune response; and

(b) administering to said patient an Eta-1/osteopontin inhibitory modulator such that the type-1 immune response is downregulated.

12. The method of claim 11, wherein the disorder is selected from the group consisting of bacterial arthritis, granulomatous disorder, glomerulonephritis, rheumatoid arthritis, multiple sclerosis, herpes simplex keratitis, and autoimmune disease.

13. A method of enhancing production of a type-1 immune response-associated cytokine by an immune cell comprising contacting said cell with an Eta-1/osteopontin stimulatory modulator such that production of the cytokine is enhanced.

14. The method of claim 13, wherein the type-1 immune response-associated cytokine is selected from the group consisting of interleukin-2 (IL-2), interleukin-12 (IL-12) and interferon-γ (IFN-γ).

15. A method of downregulating production of a type-2 immune response-associated cytokine by an immune cell comprising contacting said cell with an Eta-1/osteopontin inhibitory modulator such that production of the cytokine is downregulated.

16. The method of claim 15, wherein the type-2 immune response-associated cytokine is selected from the group consisting of interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6), and interleukin-10 (IL-10).

17. The method of claim 13 or 15, wherein the cell is a human cell.

18. The method of claim 13, wherein the immune cell is contacted in vivo.

19. The method of claim 13, wherein the immune cell is contacted ex vivo.

20. The method of claim 13, wherein the immune cell is selected from the group consisting of a macrophage, a dendritic cell, a T cell, a B cell, a monocyte and a neutrophil.

21. A method for stimulating interleukin-12 (IL-12) production by a macrophage comprising contacting said macrophage with an Eta-1/osteopontin stimulatory modulator such that production of IL-12 is stimulated.

22. A method for inhibiting interleukin-10 (IL-10) production by a macrophage comprising contacting said macrophage with an Eta-1/osteopontin stimulatory modulator such that production of IL-10 is inhibited.

23. A method for potentiating a type-1 immune response in a subject comprising:

(a) culturing immune effector cells isolated from said subject in the presence of an Eta-1/osteopontin stimulatory modulator; and

(b) administering the cultured cells to said subject such that the type-1 immune response in said subject is potentiated.

24. Modified tumor cells comprising irradiated tumor cells transduced with Eta-1/osteopontin.

25. The modified tumor cells of claim 24, wherein the cells are further transduced with GMCSF.

26. The modified tumor cells of claim 24, further comprising a pharmaceutically acceptable carrier.

27. The method of claim 1, wherein said Eta-1/osteopontin modulator is selected from the group consisting of an isolated Eta-1/osteopontin polypeptide, a biologically active fragment of an Eta-1/osteopontin polypeptide, an isolated nucleic acid molecule which encodes an Eta-1/osteopontin polypeptide and an isolated nucleic acid molecule which encodes a biologically active fragment of an Eta-1/osteopontin polypeptide.

28. The method of claim 27, wherein said Eta-1/osteopontin modulator is an isolated Eta-1/osteopontin polypeptide or biologically active fragment thereof.

29. The method of claim 28, wherein said Eta-1/osteopontin polypeptide is a human Eta-1/osteopontin polypeptide.

30. The method of claim 28, wherein said Eta-1/osteopontin polypeptide is at least 90% identical to a polypeptide having the amino acid sequence of SEQ ID NO:2.

31. The method of claim 27, wherein said Eta-1/osteopontin polypeptide comprises an amino acid sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, and SEQ ID NO:6.

32. The method of claim 27, wherein said Eta-1/osteopontin modulator is an isolated nucleic acid molecule encoding an Eta-1/osteopontin polypeptide or biologically active fragment thereof.

33. The method of claim 32, wherein said nucleic acid molecule is at least 90% identical to a nucleic acid molecule having the nucleotide sequence of SEQ ID NO:1.

34. The method of claim 32, wherein said nucleic acid molecule comprises a nucleotide sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3 and SEQ ID NO:5.

35. The method of claim 28, wherein the Eta-1/osteopontin modulator is a biologically active fragment of Eta-1/osteopontin.

36. The method of claim 32, wherein the Eta-1/osteopontin modulator is a nucleic acid molecule encoding a biologically active fragment of Eta-1/osteopontin.

37. The method of claim 35 or 36, wherein said biologically active fragment consists essentially of an IL-12 stimulatory domain of Eta-1/osteopontin.

38. The method of claim 37, wherein said IL-12 stimulatory domain comprises an amino acid sequence between 65 and 160 amino acids in length and is at least 90% identical to amino acids 71 to 168 of SEQ ID NO:2.

39. The method of claim 35 or 36, wherein said biologically active fragment consists essentially of an IL-10 inhibitory domain of Eta-1/osteopontin.

40. The method of claim 35, wherein said IL-10 inhibitory domain comprises an amino acid sequence between 65 and 160 amino acids in length and is at least 90% identical to amino acids 169-266 of SEQ ID NO:2.

41. The method of claim 1, wherein said Eta-1/osteopontin modulator is selected from the group consisting of a compound which specifically binds an Eta-1/osteopontin polypeptide, a compound which specifically binds an Eta-1/osteopontin target molecule, a compound which specifically modulates the activity of an Eta-1/osteopontin polypeptide and a compound which specifically modulates the activity of an Eta-1/osteopontin target molecule.

42. The method of claim 41, wherein said Eta-1/osteopontin modulator is an antibody which specifically binds Eta-1/osteopontin.

43. The method of claim 1, wherein said Eta-1/osteopontin modulator is a biosynthetic immunomodulatory molecule.

44. A biosynthetic immunomodulatory molecule comprising an IL-12 stimulatory component and a first biomodular component, forming a molecule which modulates an immune response.

45. The immunomodulatory molecule of claim 44, wherein the IL-12 stimulatory component is derived from Eta-1/osteopontin.

46. The immunomodulatory molecule of claim 45, wherein the IL-12 stimulatory component is a polypeptide.

47. The immunomodulatory molecule of claim 46, wherein the IL-12 stimulatory component comprises an amino acid sequence between 65 and 160 amino acid residues in length and is at least 90% identical to amino acids 71 to 168 of SEQ ID NO:2.

48. The immunomodulatory molecule of claim 46, wherein the IL-12 stimulatory component comprises amino acids 71-168 of SEQ ID NO:2.

49. A biosynthetic immunomodulatory molecule comprising an IL-10 inhibitory component and a first biomodular component, forming a molecule which modulates an immune response.

50. The immunomodulatory molecule of claim 49, wherein the IL-10 inhibitory component is derived from Eta-1/osteopontin.

51. The immunomodulatory molecule of claim 50, wherein the IL-10 inhibitory component is a polypeptide.

52. The immunomodulatory molecule of claim 50, wherein the IL-10 inhibitory component comprises an amino acid sequence between 65 and 160 amino acid residues in length and is at least 90% identical to amino acids 169 to 266 of SEQ ID NO:2.

53. The immunomodulatory molecule of claim 47, wherein the IL-10 inhibitory component comprises amino acids 169 to 266 of SEQ ID NO:2.

54. The immunomodulatory molecule of claim 44 or 49, wherein the first biomodular component is selected from the group consisting of a signal peptide, a calcium/apatite binding domain and a heparin binding domain.

55. The biosynthetic immunomodulatory molecule of claim 44 or 49, further comprising a second biomodular component.

56. The immunomodulatory molecule of claim 55, wherein the second biomodular component is selected from the group consisting of a signal peptide, a calcium/apatite binding domain and a heparin binding domain.

57. A biosynthetic immunomodulatory molecule comprising an IL-12 stimulatory component, a calcium/apatite binding domain and and a heparin binding domain.

58. A biosynthetic immunomodulatory molecule comprising an IL-10 inhibitory component, a signal peptide, a calcium/apatite binding domain and a heparin binding domain.

59. The immunomodulatory molecule of claim 44 or 49, wherein the molecule modulates an immune response selected from the group consisting of modulation of cytokine secretion, regulation of chemotaxis, regulation of hapotaxis, and regulation of cell spreading.

60. A biosynthetic immunomodulatory molecule comprising the amino acid sequence of SEQ ID NO:8.

61. A biosynthetic immunomodulatory molecule comprising the amino acid sequence of SEQ ID NO:10.

62. A biosynthetic immunomodulatory molecule encoded by nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO:7.

63. A biosynthetic immunomodulatory molecule encoded by nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO:9.

64. An isolated nucleic acid molecule comprising nucleic acid sequences which encode the immunomodulatory molecule of claim 44 or 49.

65. An expression vector comprising the nucleic acid molecule of claim 64.

66. A host cell comprising the vector of claim 65.

67. A method of producing an immunomodulatory molecule, comprising culturing the host cell of claim 66 under conditions such that the immunomodulatory molecule is produced.

68. A pharmaceutical composition comprising the immunomodulatory molecule of claim 44 or 49, and a pharmaceutically acceptable carrier.

69. A method of modulating an immune response in a cell comprising contacting the cell with an immunomodulatory molecule of claim 44 or 49 such that an immune response is modulated.

70. The method of claim 69, wherein the cell is present within a subject and the immunomodulatory molecule is administered to the subject.

FIGURE 1

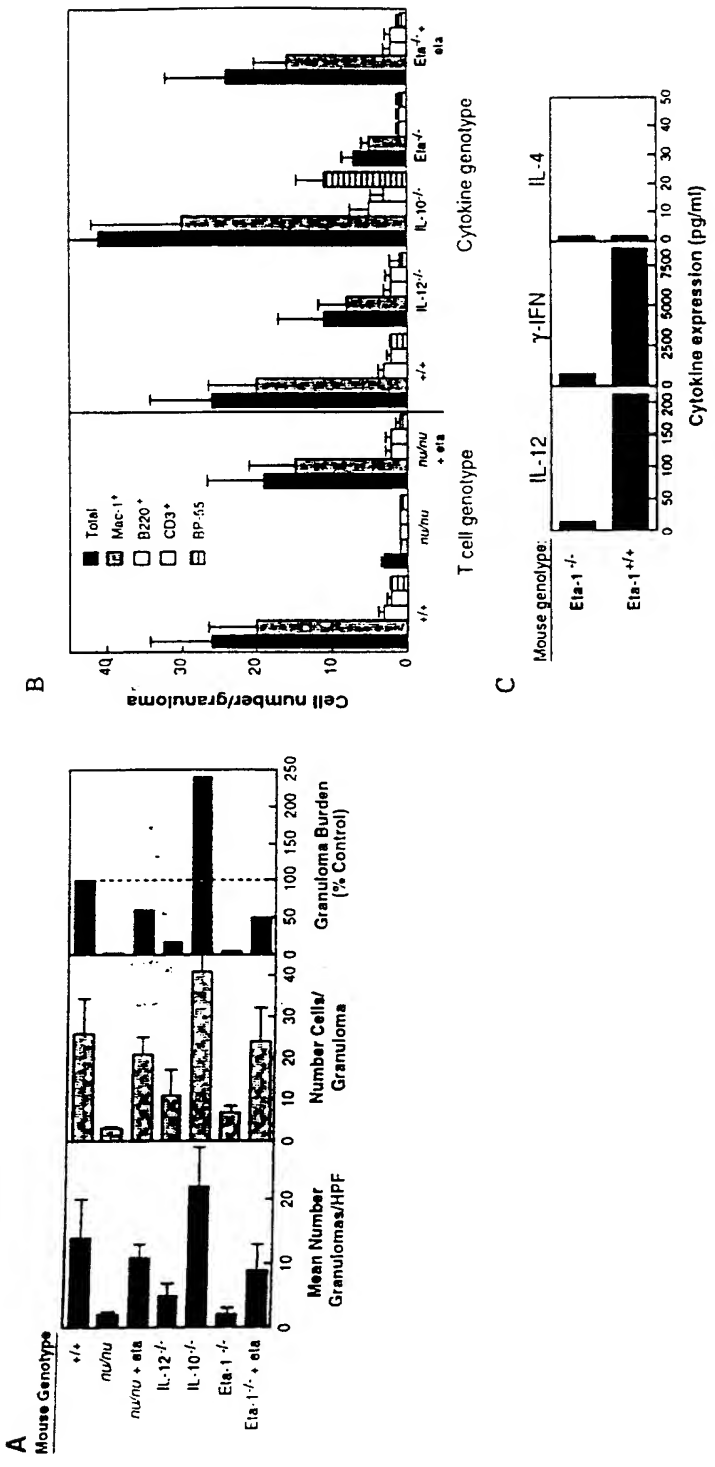
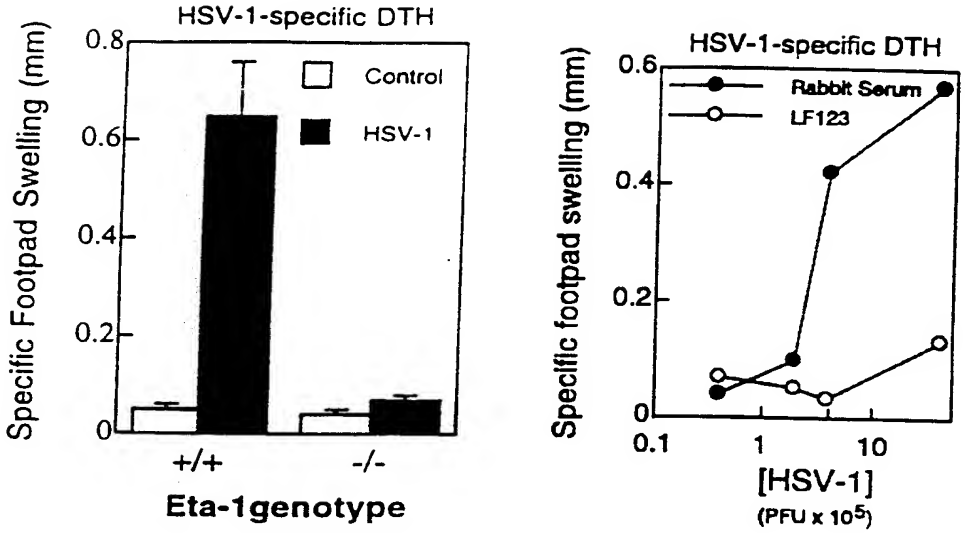

A
Mouse Genotype
+/+
nu/nu
nu/nu + eta
IL-12 -/-
IL-10 -/-
Eta-1 -/-
Eta-1 -/- + eta
Mean Number Granulomas/HPF
Number Cells/ Granuloma
Granuloma Burden (% Control)
B
Cell number/granuloma
Total
Mac-1+
B220+
CD3+
BP-55
T cell genotype
Cytokine genotype
C
Mouse genotype:
IL-12
γ-IFN
IL-4
Eta-1 -/-
Eta-1 +/+
Cytokine expression (pg/ml)

FIGURE 2

A


HSV-1-specific DTH
Specific Footpad Swelling (mm)
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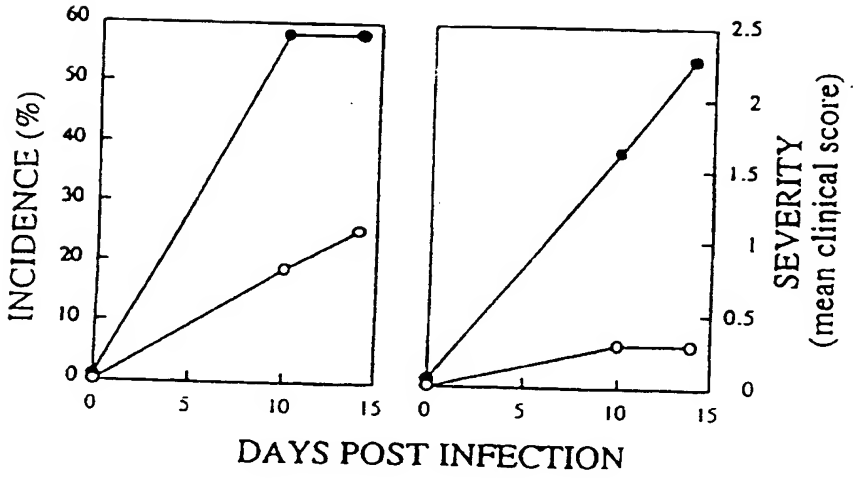
0.6
0.4
0.2
0
Control
HSV-1
+/+
-/-
Eta-1genotype
HSV-1-specific DTH
Specific footpad swelling (mm)
0.6
0.4
0.2
0
Rabbit Serum
LF123
0.1
1
10
[HSV-1]
(PFU x 10^5)

C


INCIDENCE (%)
60
50
40
30
20
10
0
0
5
10
15
2.5
2
1.5
1
0.5
0
SEVERITY
(mean clinical score)
DAYS POST INFECTION

D

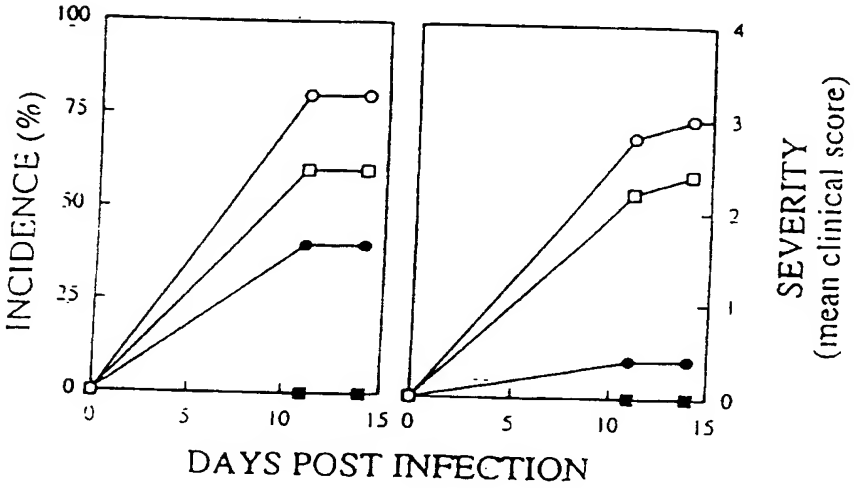

INCIDENCE (%)
100
75
50
25
0
0
5
10
15
4
3
2
1
0
SEVERITY
(mean clinical score)
DAYS POST INFECTION

FIGURE 2 (Continued)

E

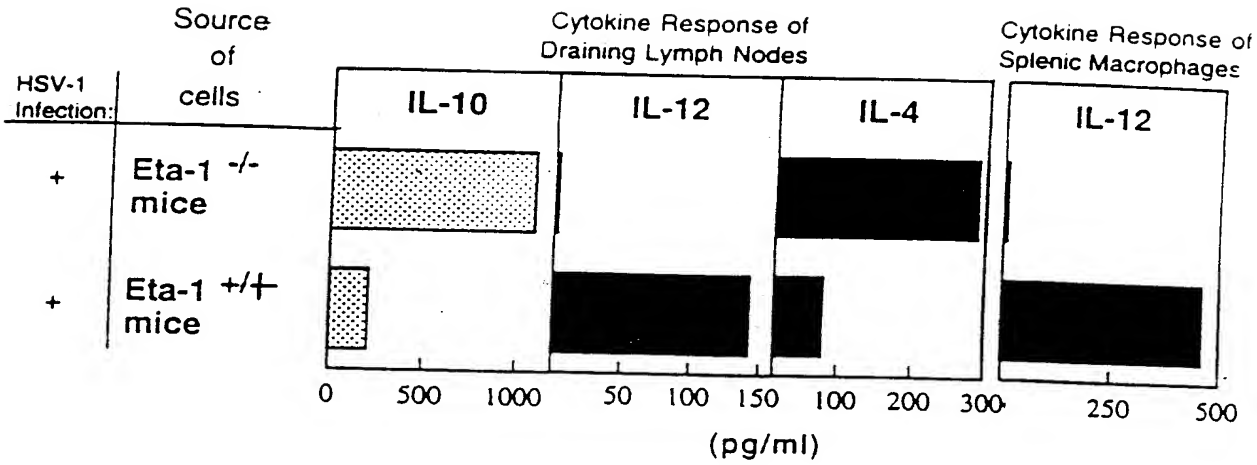


FIGURE 3

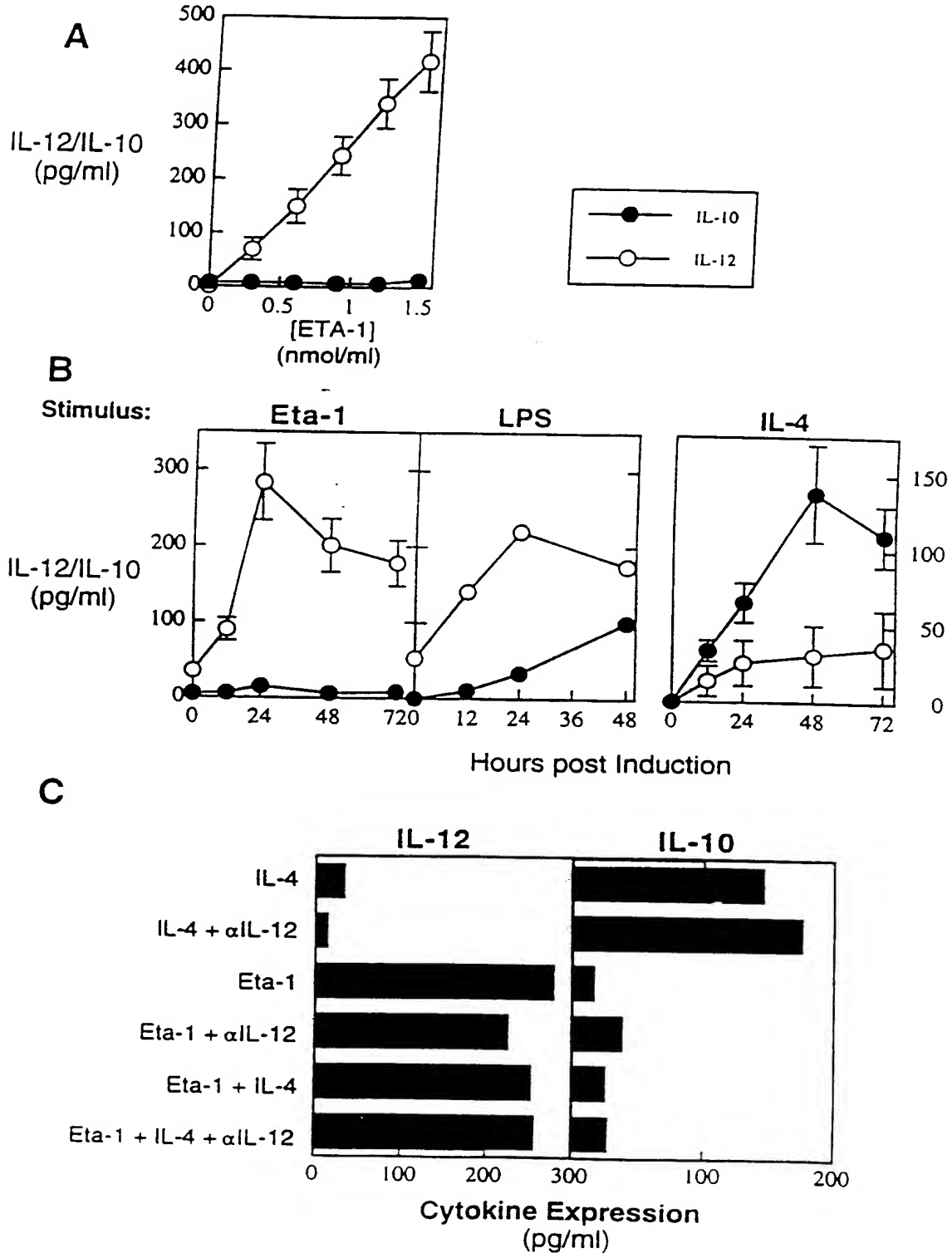

A
IL-12/IL-10
(pg/ml)
500
400
300
200
100
0
0
0.5
1
1.5
[ETA-1]
(nmol/ml)
IL-10
IL-12
B
Stimulus:
Eta-1
LPS
IL-4
IL-12/IL-10
(pg/ml)
300
200
100
0
0
24
48
720
12
24
36
48
0
24
48
72
150
100
50
0
Hours post Induction
C
IL-12
IL-10
IL-4
IL-4 + αIL-12
Eta-1
Eta-1 + αIL-12
Eta-1 + IL-4
Eta-1 + IL-4 + αIL-12
0
100
200
300
100
200
Cytokine Expression
(pg/ml)

FIGURE 3 (Continued)

D

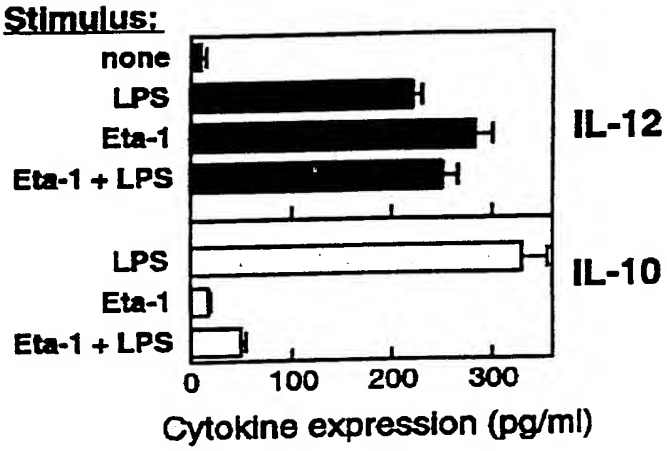
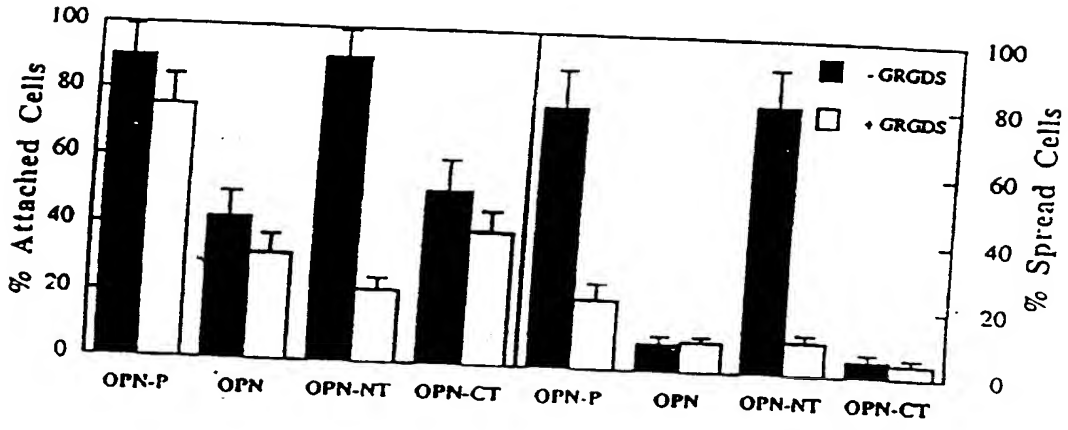


FIGURE 4

A


% Attached Cells
100
80
60
40
20
0
- GRGDS
+ GRGDS
% Spread Cells
100
80
60
40
20
0
OPN-P
OPN
OPN-NT
OPN-CT
OPN-P
OPN
OPN-NT
OPN-CT

B

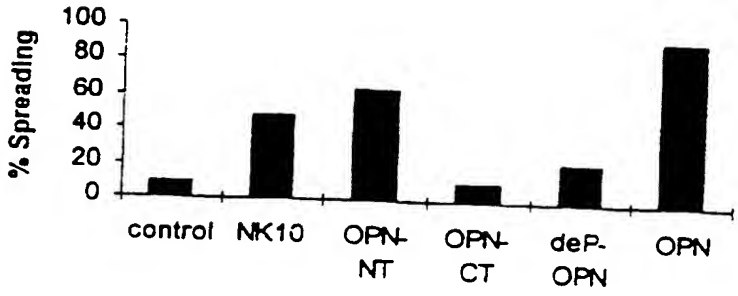

% Spreading
100
80
60
40
20
0
control
NK10
OPN-NT
OPN-CT
deP-OPN
OPN

FIGURE 5

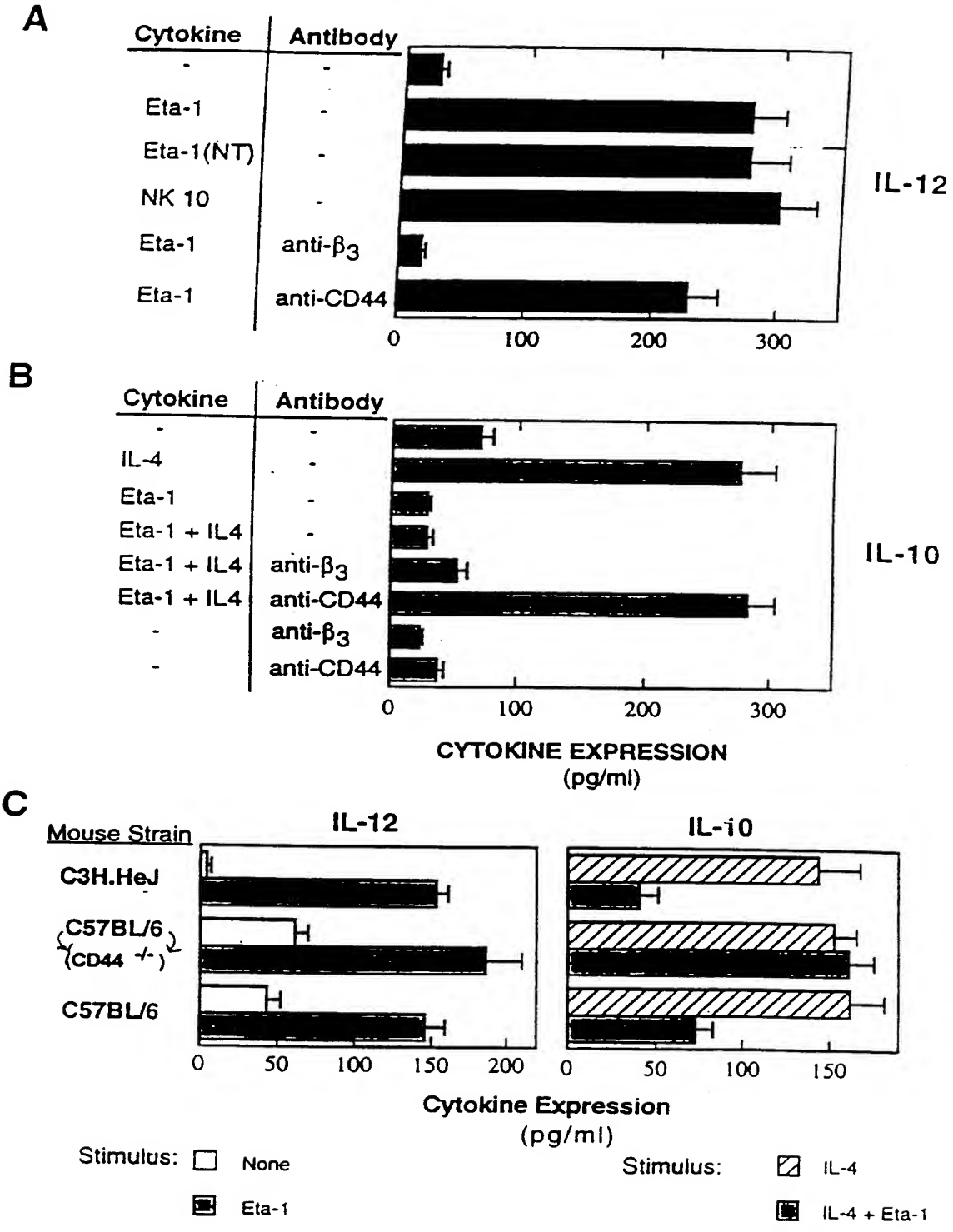

A
Cytokine
Antibody
-
-
Eta-1
-
Eta-1(NT)
-
NK 10
-
Eta-1
anti-β3
Eta-1
anti-CD44
0
100
200
300
IL-12
B
Cytokine
Antibody
-
-
IL-4
-
Eta-1
-
Eta-1 + IL4
-
Eta-1 + IL4
anti-β3
Eta-1 + IL4
anti-CD44
-
anti-β3
-
anti-CD44
0
100
200
300
IL-10
CYTOKINE EXPRESSION
(pg/ml)
C
Mouse Strain
IL-12
IL-10
C3H.HeJ
C57BL/6
(CD44 -/-)
C57BL/6
0
50
100
150
200
0
50
100
150
Cytokine Expression
(pg/ml)
Stimulus:
None
Eta-1
Stimulus:
IL-4
IL-4 + Eta-1

FIGURE 6

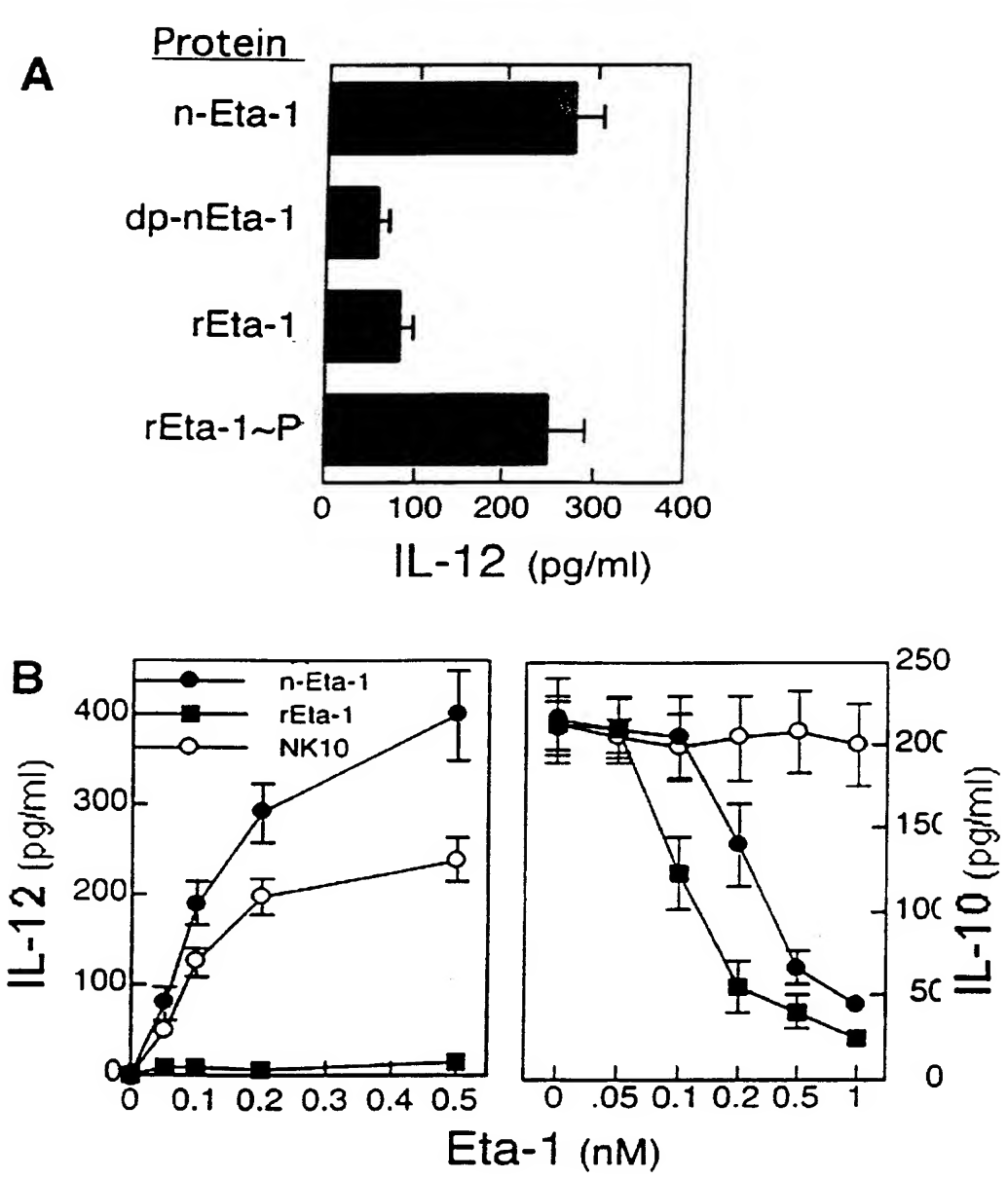

A
Protein
n-Eta-1
dp-nEta-1
rEta-1
rEta-1~P
0 100 200 300 400
IL-12 (pg/ml)
B
n-Eta-1
rEta-1
NK10
IL-12 (pg/ml)
400
300
200
100
0
0 0.1 0.2 0.3 0.4 0.5
250
200
150
100
50
0
0 .05 0.1 0.2 0.5 1
IL-10 (pg/ml)
Eta-1 (nM)

FIGURE 7

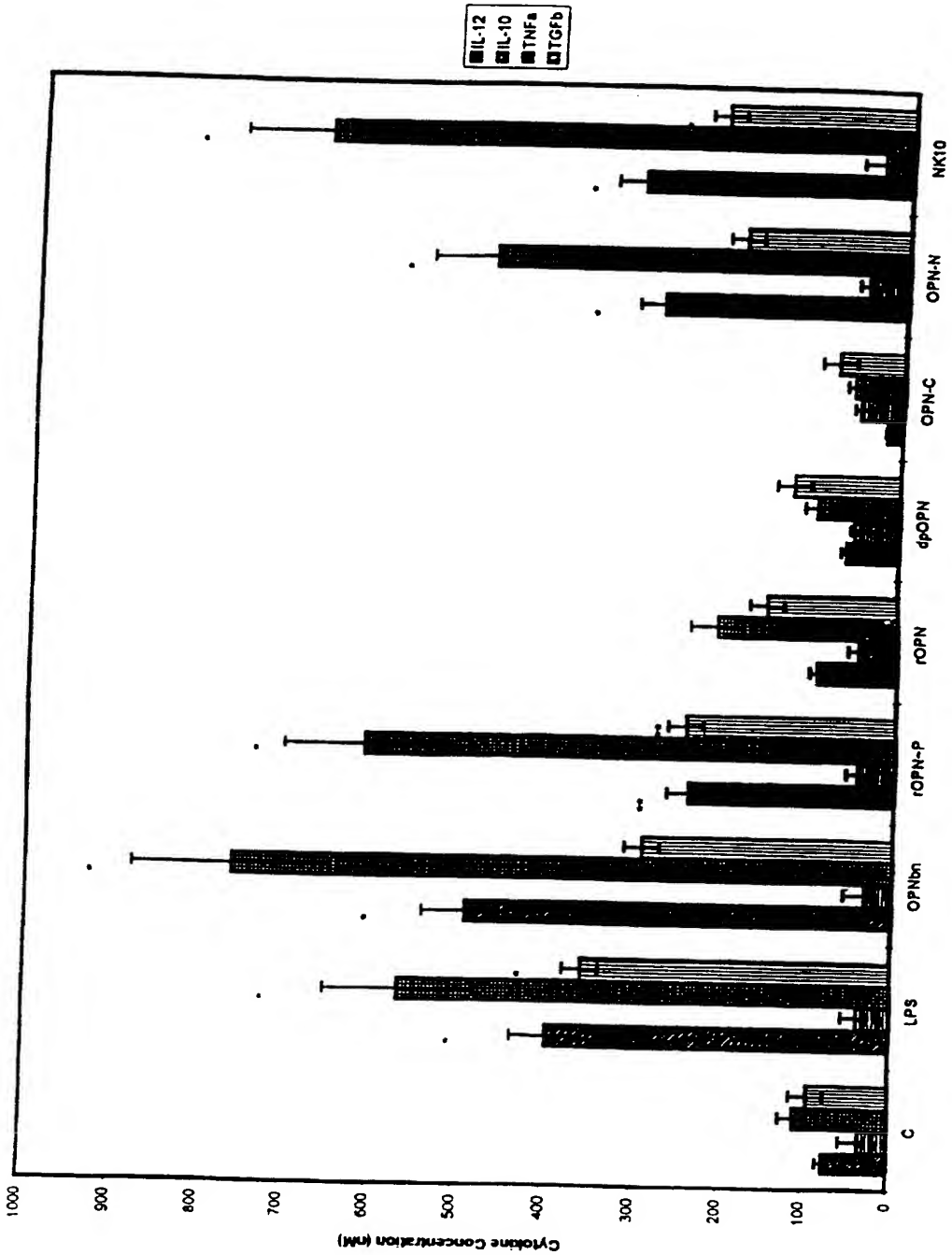

IL-12
IL-10
TNFa
TGFb
NK10
OPN-N
OPN-C
dpOPN
rOPN
rOPN-P
OPNbn
LPS
C
1000
900
800
700
600
500
400
300
200
100
0
Cytokine Concentration (nM)

FIGURE 8

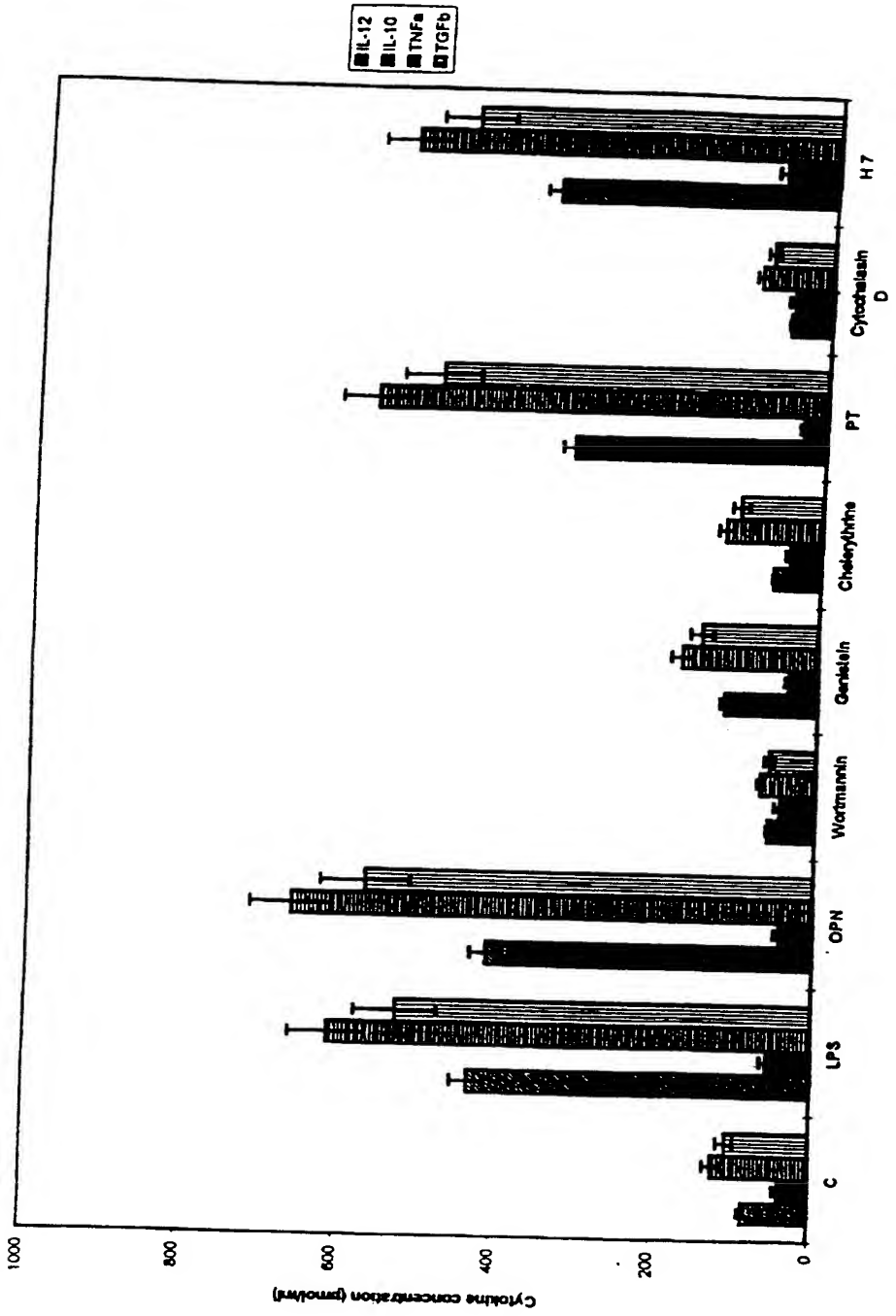

IL-12
IL-10
TNFa
TGFb
H7
Cytochalasin D
PT
Chelerythrine
Genistein
Wortmannin
OPN
LPS
C
0
200
400
600
800
1000
Cytokine concentration (pmol/ml)

FIGURE 9

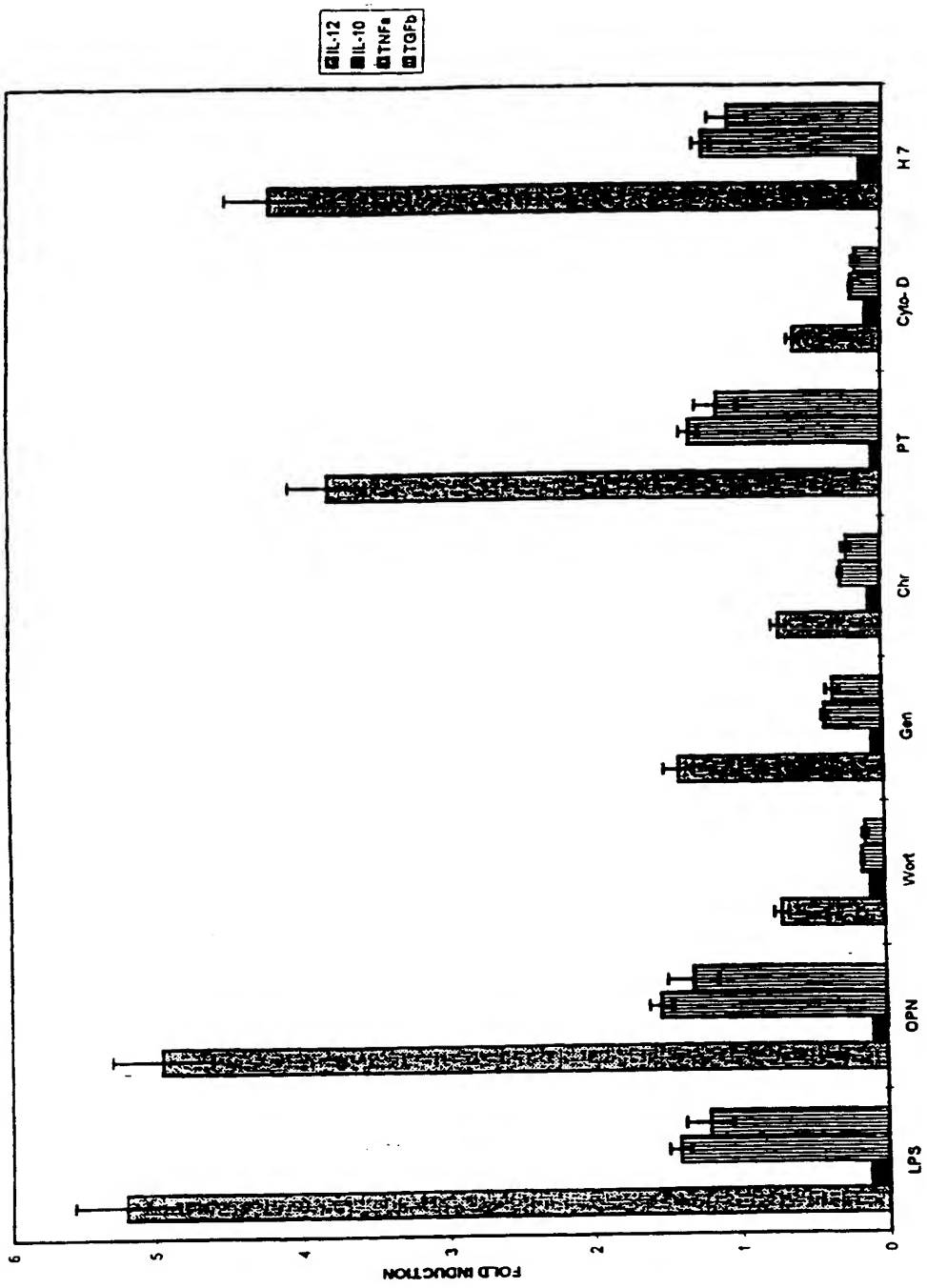

IL-12
IL-10
TNFa
TGFb
H7
Cyto-D
PT
Chr
Gen
Wort
OPN
LPS
FOLD INDUCTION
0
1
2
3
4
5
6

FIG.10

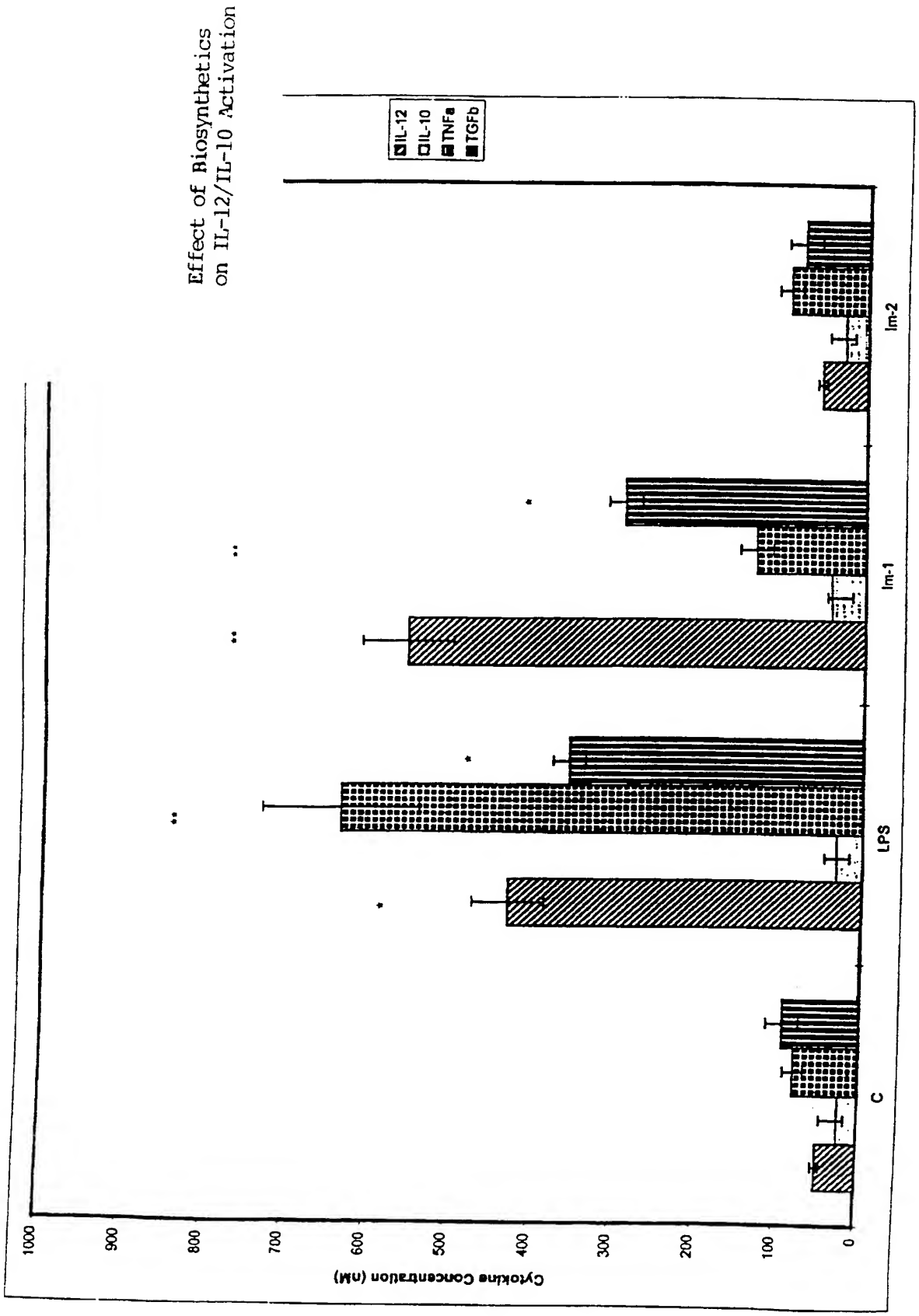
```
   agaattgcagtgatttgcttttgcctcctaggcatcacctgt
      R  I  A  V  I  C  F  C  L  L  G  I  T  C
   gccataccagttaaacaggctgattctggaagttctgaggaattc
   A  I  P  V  K  Q  A  D  S  G  S  S  E  E  F
Gatgatgaggagctctctcaccattctgatgaatctgatgaactggtcactgat
D  D  D  D  S  H  H  S  D  E  S  D  E  L  V  T  D

tttcccacggacctgccagcaaccgaagttttcactccagttgtc
F  P  T  D  L  P  A  T  E  V  F  T  P  V  V
cccacagtagacacatatgatggccgaggtgatagtgtggtttat
P  T  V  D  T  Y  D  G  R  G  D  S  V  V  Y
ggactgagtaa
G  L  R
```

FIG.11

```
atgagaattgcagtgatttgcttttgcctcctaggcatcacctgt
 M  R  I  A  V  I  C  F  C  L  L  G  I  T  C
gccataccagttaaacaggctgattctggaagttctgaggaaatg
 A  I  P  V  K  Q  A  D  S  G  S  S  E  E  M
catcaccaccatcaccatctcaggtcaaaatctaagaagtttcgc
 H  H  H  H  H  H  L  R  S  K  S  K  K  F  R
agacctgacatccagtaccctgatgctacagacgaggacatcacc
 R  P  D  I  Q  Y  P  D  A  T  D  E  D  I  T
tcacacatggaaagcgaggagttgaatggtgcatacaaggccatc
 S  H  M  E  S  E  E  L  N  G  A  Y  K  A  I
cccgttgcccaggacctgaacgcgccttctgattgggacagccgt
 P  V  A  Q  D  L  N  A  P  S  D  W  D  S  R
gggaaggacagttatgaaacgagtcagctggatgaccagagtgct
 G  K  D  S  Y  E  T  S  Q  L  D  D  Q  S  A
gaaacccacagccacaagcagtccagattatataagcggaaagcc
 E  T  H  S  H  K  Q  S  R  L  Y  K  R  K  A
aatgatgagagcaatgagcattccgatgtgattgatagtcaggaa
 N  D  E  S  N  E  H  S  D  V  I  D  S  Q  E
ctttccaaagtcagccgtgaattccacagccatgaatttcacagc
 L  S  K  V  S  R  E  F  H  S  H  E  F  H  S
catgaagatatgctggttgtagaccccaaaagtaaggaagaagat
 H  E  D  M  L  V  V  D  P  K  S  K  E  E  D
aaacacctgaaatttcgtatttctcatgaattagatagtgcatct
 K  H  L  K  F  R  I  S  H  E  L  D  S  A  S
tctgaggtcaat
 S  E  V  N
```

FIGURE 12



im1and2 cyto Chart 1

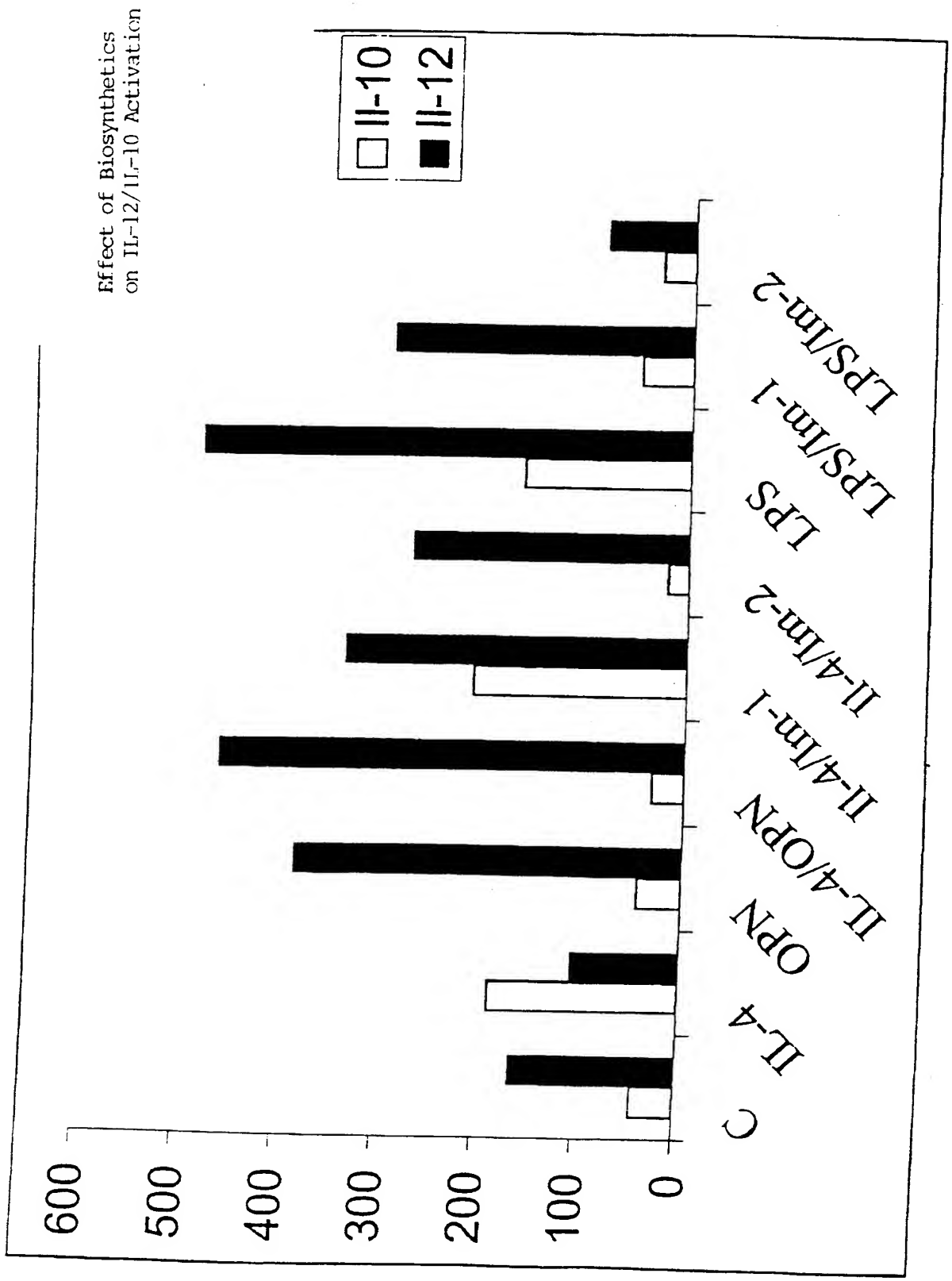
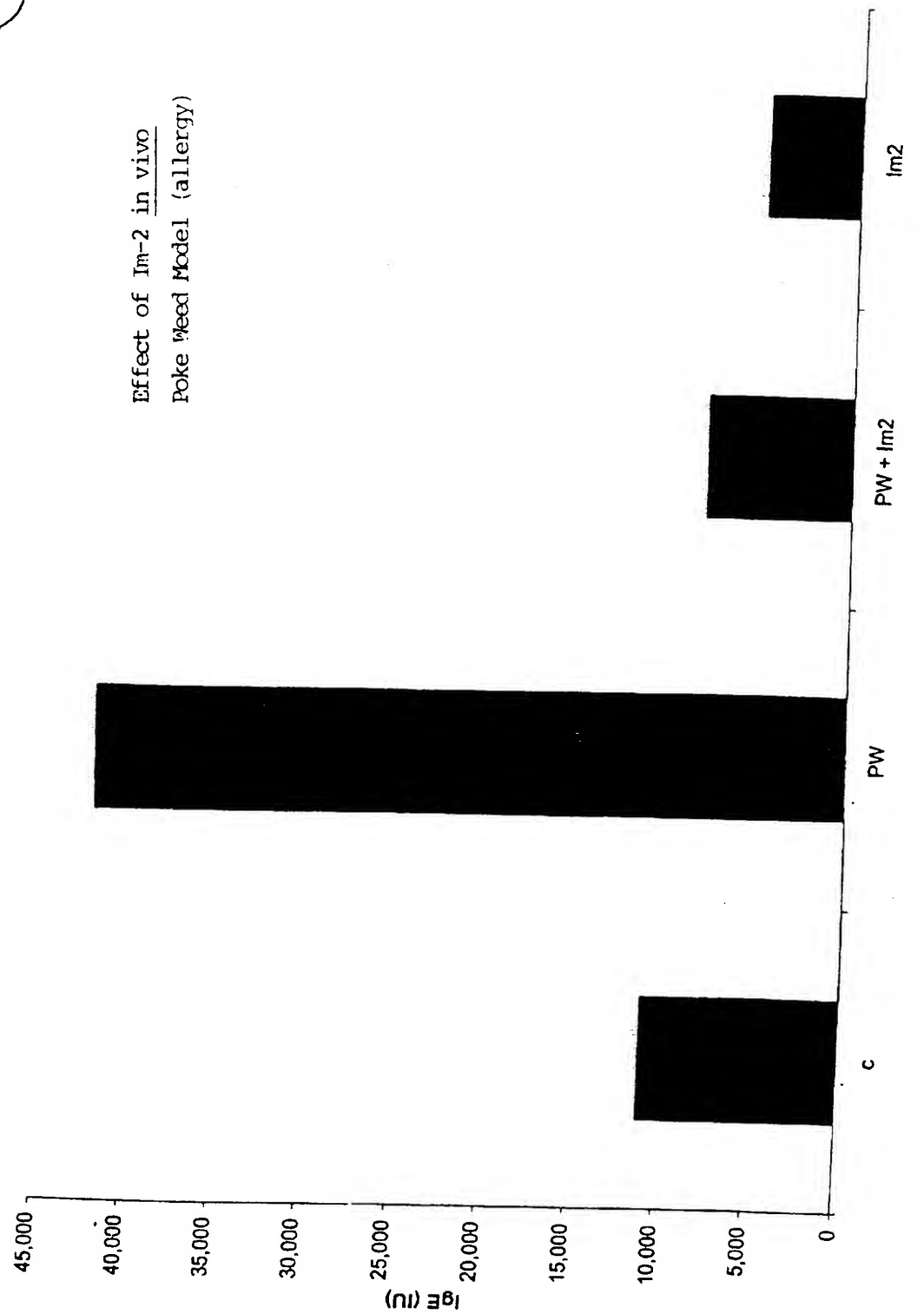

Effect of Biosynthetics
on IL-12/IL-10 Activation
IL-10
IL-12
600
500
400
300
200
100
0
C
IL-4
OPN
IL-4/OPN
IL-4/Im-1
IL-4/Im-2
LPS
LPS/Im-1
LPS/Im-2

FIGURE 13

01

FIGURE 14

Inhibition of IgE Production by Im-2



SEQUENCE LISTING

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CHILDREN'S MEDICAL CENTER CORPORATION

<120> METHODS AND COMPOSITIONS FOR MODULATING AN IMMUNE RESPONSE

<130> CMZ-121CPPC

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ata cca gtt aaa cag gct gat tct gga agt tct gag gaa aag cag ctt   96
Ile Pro Val Lys Gln Ala Asp Ser Gly Ser Ser Glu Glu Lys Gln Leu
             20                  25                  30

tac aac aaa tac cca gat gct gtg gcc aca tgg cta aac cct gac cca   144
Tyr Asn Lys Tyr Pro Asp Ala Val Ala Thr Trp Leu Asn Pro Asp Pro
         35                  40                  45

tct cag aag cag aat ctc cta gcc cca cag aat gct gtg tcc tct gaa   192
Ser Gln Lys Gln Asn Leu Leu Ala Pro Gln Asn Ala Val Ser Ser Glu
     50                  55                  60

gaa acc aat gac ttt aaa caa gag acc ctt cca agt aag tcc aac gaa   240
Glu Thr Asn Asp Phe Lys Gln Glu Thr Leu Pro Ser Lys Ser Asn Glu
 65                  70                  75                  80

agc cat gac cac atg gat gat atg gat gat gaa gat gat gat gac cat   288
Ser His Asp His Met Asp Asp Met Asp Asp Glu Asp Asp Asp Asp His
                 85                  90                  95

gtg gac agc cag gac tcc att gac tcg aac gac tct gat gat gta gat   336
Val Asp Ser Gln Asp Ser Ile Asp Ser Asn Asp Ser Asp Asp Val Asp
            100                 105                 110
```

```
gac act gat gat tct cac cag tct gat gag tct cac cat tct gat gaa   384
Asp Thr Asp Asp Ser His Gln Ser Asp Glu Ser His His Ser Asp Glu
        115                 120                 125

tct gat gaa ctg gtc act gat ttt ccc acg gac ctg cca gca acc gaa   432
Ser Asp Glu Leu Val Thr Asp Phe Pro Thr Asp Leu Pro Ala Thr Glu
    130                 135                 140

gtt ttc act cca gtt gtc ccc aca gta gac aca tat gat ggc cga ggt   480
Val Phe Thr Pro Val Val Pro Thr Val Asp Thr Tyr Asp Gly Arg Gly
145                 150                 155                 160

gat agt gtg gtt tat gga ctg agg tca aaa tct aag aag ttt cgc aga   528
Asp Ser Val Val Tyr Gly Leu Arg Ser Lys Ser Lys Lys Phe Arg Arg
                165                 170                 175

cct gac atc cag tac cct gat gct aca gac gag gac atc acc tca cac   576
Pro Asp Ile Gln Tyr Pro Asp Ala Thr Asp Glu Asp Ile Thr Ser His
            180                 185                 190

atg gaa agc gag gag ttg aat ggt gca tac aag gcc atc ccc gtt gcc   624
Met Glu Ser Glu Glu Leu Asn Gly Ala Tyr Lys Ala Ile Pro Val Ala
        195                 200                 205

cag gac ctg aac gcg cct tct gat tgg gac agc cgt ggg aag gac agt   672
Gln Asp Leu Asn Ala Pro Ser Asp Trp Asp Ser Arg Gly Lys Asp Ser
    210                 215                 220

tat gaa acg agt cag ctg gat gac cag agt gct gaa acc cac agc cac   720
Tyr Glu Thr Ser Gln Leu Asp Asp Gln Ser Ala Glu Thr His Ser His
225                 230                 235                 240

aag cag tcc aga tta tat aag cgg aaa gcc aat gat gag agc aat gag   768
Lys Gln Ser Arg Leu Tyr Lys Arg Lys Ala Asn Asp Glu Ser Asn Glu
                245                 250                 255

cat tcc gat gtg att gat agt cag gaa ctt tcc aaa gtc agc cgt gaa   816
His Ser Asp Val Ile Asp Ser Gln Glu Leu Ser Lys Val Ser Arg Glu
            260                 265                 270

ttc cac agc cat gaa ttt cac agc cat gaa gat atg ctg gtt gta gac   864
Phe His Ser His Glu Phe His Ser His Glu Asp Met Leu Val Val Asp
        275                 280                 285

ccc aaa agt aag gaa gaa gat aaa cac ctg aaa ttt cgt att tct cat   912
Pro Lys Ser Lys Glu Glu Asp Lys His Leu Lys Phe Arg Ile Ser His
    290                 295                 300

gaa tta gat agt gca tct tct gag gtc aat taa                       945
Glu Leu Asp Ser Ala Ser Ser Glu Val Asn
305                 310
```

<210> 2
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<212> PRT
<213> Homo sapiens

<400> 2

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Met Arg Ile Ala Val Ile Cys Phe Cys Leu Leu Gly Ile Thr Cys Ala
 1               5                  10                  15

Ile Pro Val Lys Gln Ala Asp Ser Gly Ser Ser Glu Glu Lys Gln Leu
            20                  25                  30

Tyr Asn Lys Tyr Pro Asp Ala Val Ala Thr Trp Leu Asn Pro Asp Pro
        35                  40                  45

Ser Gln Lys Gln Asn Leu Leu Ala Pro Gln Asn Ala Val Ser Ser Glu
    50                  55                  60

Glu Thr Asn Asp Phe Lys Gln Glu Thr Leu Pro Ser Lys Ser Asn Glu
65                  70                  75                  80

Ser His Asp His Met Asp Asp Met Asp Asp Glu Asp Asp Asp Asp His
                85                  90                  95

Val Asp Ser Gln Asp Ser Ile Asp Ser Asn Asp Ser Asp Asp Val Asp
            100                 105                 110

Asp Thr Asp Asp Ser His Gln Ser Asp Glu Ser His His Ser Asp Glu
        115                 120                 125

Ser Asp Glu Leu Val Thr Asp Phe Pro Thr Asp Leu Pro Ala Thr Glu
    130                 135                 140

Val Phe Thr Pro Val Val Pro Thr Val Asp Thr Tyr Asp Gly Arg Gly
145                 150                 155                 160

Asp Ser Val Val Tyr Gly Leu Arg Ser Lys Ser Lys Lys Phe Arg Arg
                165                 170                 175

Pro Asp Ile Gln Tyr Pro Asp Ala Thr Asp Glu Asp Ile Thr Ser His
            180                 185                 190

Met Glu Ser Glu Glu Leu Asn Gly Ala Tyr Lys Ala Ile Pro Val Ala
        195                 200                 205

Gln Asp Leu Asn Ala Pro Ser Asp Trp Asp Ser Arg Gly Lys Asp Ser
    210                 215                 220

Tyr Glu Thr Ser Gln Leu Asp Asp Gln Ser Ala Glu Thr His Ser His
225                 230                 235                 240

Lys Gln Ser Arg Leu Tyr Lys Arg Lys Ala Asn Asp Glu Ser Asn Glu
                245                 250                 255

His Ser Asp Val Ile Asp Ser Gln Glu Leu Ser Lys Val Ser Arg Glu
            260                 265                 270

Phe His Ser His Glu Phe His Ser His Glu Asp Met Leu Val Val Asp
        275                 280                 285

Pro Lys Ser Lys Glu Glu Asp Lys His Leu Lys Phe Arg Ile Ser His
    290                 295                 300
```

```
Glu Leu Asp Ser Ala Ser Ser Glu Val Asn
305                 310


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<213> Homo sapiens

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Met Arg Ile Ala Val Ile Cys Phe Cys Leu Leu Gly Ile Thr Cys Ala
  1               5                  10                  15

ata cca gtt aaa cag gct gat tct gga agt tct gag gaa aag cag ctt   96
Ile Pro Val Lys Gln Ala Asp Ser Gly Ser Ser Glu Glu Lys Gln Leu
             20                  25                  30

tac aac aaa tac cca gat gct gtg gcc aca tgg cta aac cct gac cca   144
Tyr Asn Lys Tyr Pro Asp Ala Val Ala Thr Trp Leu Asn Pro Asp Pro
         35                  40                  45

tct cag aag cag aat ctc cta gcc cca cag acc ctt cca agt aag tcc   192
Ser Gln Lys Gln Asn Leu Leu Ala Pro Gln Thr Leu Pro Ser Lys Ser
     50                  55                  60

aac gaa agc cat gac cac atg gat gat atg gat gat gaa gat gat gat   240
Asn Glu Ser His Asp His Met Asp Asp Met Asp Asp Glu Asp Asp Asp
 65                  70                  75                  80

gac cat gtg gac agc cag gac tcc att gac tcg aac gac tct gat gat   288
Asp His Val Asp Ser Gln Asp Ser Ile Asp Ser Asn Asp Ser Asp Asp
                 85                  90                  95

gta gat gac act gat gat tct cac cag tct gat gag tct cac cat tct   336
Val Asp Asp Thr Asp Asp Ser His Gln Ser Asp Glu Ser His His Ser
            100                 105                 110

gat gaa tct gat gaa ctg gtc act gat ttt ccc acg gac ctg cca gca   384
Asp Glu Ser Asp Glu Leu Val Thr Asp Phe Pro Thr Asp Leu Pro Ala
        115                 120                 125

acc gaa gtt ttc act cca gtt gtc ccc aca gta gac aca tat gat ggc   432
Thr Glu Val Phe Thr Pro Val Val Pro Thr Val Asp Thr Tyr Asp Gly
    130                 135                 140

cga ggt gat agt gtg gtt tat gga ctg agg tca aaa tct aag aag ttt   480
Arg Gly Asp Ser Val Val Tyr Gly Leu Arg Ser Lys Ser Lys Lys Phe
145                 150                 155                 160

cgc aga cct gac atc cag tac cct gat gct aca gac gag gac atc acc   528
Arg Arg Pro Asp Ile Gln Tyr Pro Asp Ala Thr Asp Glu Asp Ile Thr
                165                 170                 175
```

```
tca cac atg gaa agc gag gag ttg aat ggt gca tac aag gcc atc ccc   576
Ser His Met Glu Ser Glu Glu Leu Asn Gly Ala Tyr Lys Ala Ile Pro
            180                 185                 190

gtt gcc cag gac ctg aac gcg cct tct gat tgg gac agc cgt ggg aag   624
Val Ala Gln Asp Leu Asn Ala Pro Ser Asp Trp Asp Ser Arg Gly Lys
        195                 200                 205

gac agt tat gaa acg agt cag ctg gat gac cag agt gct gaa acc cac   672
Asp Ser Tyr Glu Thr Ser Gln Leu Asp Asp Gln Ser Ala Glu Thr His
    210                 215                 220

agc cac aag cag tcc aga tta tat aag cgg aaa gcc aat gat gag agc   720
Ser His Lys Gln Ser Arg Leu Tyr Lys Arg Lys Ala Asn Asp Glu Ser
225                 230                 235                 240

aat gag cat tcc gat gtg att gat agt cag gaa ctt tcc aaa gtc agc   768
Asn Glu His Ser Asp Val Ile Asp Ser Gln Glu Leu Ser Lys Val Ser
                245                 250                 255

cgt gaa ttc cac agc cat gaa ttt cac agc cat gaa gat atg ctg gtt   816
Arg Glu Phe His Ser His Glu Phe His Ser His Glu Asp Met Leu Val
            260                 265                 270

gta gac ccc aaa agt aag gaa gaa gat aaa cac ctg aaa ttt cgt att   864
Val Asp Pro Lys Ser Lys Glu Glu Asp Lys His Leu Lys Phe Arg Ile
        275                 280                 285

tct cat gaa tta gat agt gca tct tct gag gtc aat taa               903
Ser His Glu Leu Asp Ser Ala Ser Ser Glu Val Asn
    290                 295                 300
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<212> PRT
<213> Homo sapiens

<400> 4

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Ile Pro Val Lys Gln Ala Asp Ser Gly Ser Ser Glu Glu Lys Gln Leu
             20                  25                  30

Tyr Asn Lys Tyr Pro Asp Ala Val Ala Thr Trp Leu Asn Pro Asp Pro
         35                  40                  45

Ser Gln Lys Gln Asn Leu Leu Ala Pro Gln Thr Leu Pro Ser Lys Ser
     50                  55                  60

Asn Glu Ser His Asp His Met Asp Asp Met Asp Asp Glu Asp Asp Asp
 65                  70                  75                  80

Asp His Val Asp Ser Gln Asp Ser Ile Asp Ser Asn Asp Ser Asp Asp
                85                  90                  95
```

```
Val Asp Asp Thr Asp Asp Ser His Gln Ser Asp Glu Ser His His Ser
            100                 105                 110

Asp Glu Ser Asp Glu Leu Val Thr Asp Phe Pro Thr Asp Leu Pro Ala
        115                 120                 125

Thr Glu Val Phe Thr Pro Val Val Pro Thr Val Asp Thr Tyr Asp Gly
    130                 135                 140

Arg Gly Asp Ser Val Val Tyr Gly Leu Arg Ser Lys Ser Lys Lys Phe
145                 150                 155                 160

Arg Arg Pro Asp Ile Gln Tyr Pro Asp Ala Thr Asp Glu Asp Ile Thr
                165                 170                 175

Ser His Met Glu Ser Glu Glu Leu Asn Gly Ala Tyr Lys Ala Ile Pro
            180                 185                 190

Val Ala Gln Asp Leu Asn Ala Pro Ser Asp Trp Asp Ser Arg Gly Lys
        195                 200                 205

Asp Ser Tyr Glu Thr Ser Gln Leu Asp Asp Gln Ser Ala Glu Thr His
    210                 215                 220

Ser His Lys Gln Ser Arg Leu Tyr Lys Arg Lys Ala Asn Asp Glu Ser
225                 230                 235                 240

Asn Glu His Ser Asp Val Ile Asp Ser Gln Glu Leu Ser Lys Val Ser
                245                 250                 255

Arg Glu Phe His Ser His Glu Phe His Ser His Glu Asp Met Leu Val
            260                 265                 270

Val Asp Pro Lys Ser Lys Glu Glu Asp Lys His Leu Lys Phe Arg Ile
        275                 280                 285

Ser His Glu Leu Asp Ser Ala Ser Ser Glu Val Asn
    290                 295                 300


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  1               5                  10                  15

ata cca gtt aaa cag gct gat tct gga agt tct gag gaa aag cag aat   96
Ile Pro Val Lys Gln Ala Asp Ser Gly Ser Ser Glu Glu Lys Gln Asn
            20                  25                  30
```

```
gct gtg tcc tct gaa gaa acc aat gac ttt aaa caa gag acc ctt cca   144
Ala Val Ser Ser Glu Glu Thr Asn Asp Phe Lys Gln Glu Thr Leu Pro
          35                  40                  45

agt aag tcc aac gaa agc cat gac cac atg gat gat atg gat gat gaa   192
Ser Lys Ser Asn Glu Ser His Asp His Met Asp Asp Met Asp Asp Glu
      50                  55                  60

gat gat gat gac cat gtg gac agc cag gac tcc att gac tcg aac gac   240
Asp Asp Asp Asp His Val Asp Ser Gln Asp Ser Ile Asp Ser Asn Asp
 65                  70                  75                  80

tct gat gat gta gat gac act gat gat tct cac cag tct gat gag tct   288
Ser Asp Asp Val Asp Asp Thr Asp Asp Ser His Gln Ser Asp Glu Ser
                 85                  90                  95

cac cat tct gat gaa tct gat gaa ctg gtc act gat ttt ccc acg gac   336
His His Ser Asp Glu Ser Asp Glu Leu Val Thr Asp Phe Pro Thr Asp
            100                 105                 110

ctg cca gca acc gaa gtt ttc act cca gtt gtc ccc aca gta gac aca   384
Leu Pro Ala Thr Glu Val Phe Thr Pro Val Val Pro Thr Val Asp Thr
        115                 120                 125

tat gat ggc cga ggt gat agt gtg gtt tat gga ctg agg tca aaa tct   432
Tyr Asp Gly Arg Gly Asp Ser Val Val Tyr Gly Leu Arg Ser Lys Ser
    130                 135                 140

aag aag ttt cgc aga cct gac atc cag tac cct gat gct aca gac gag   480
Lys Lys Phe Arg Arg Pro Asp Ile Gln Tyr Pro Asp Ala Thr Asp Glu
145                 150                 155                 160

gac atc acc tca cac atg gaa agc gag gag ttg aat ggt gca tac aag   528
Asp Ile Thr Ser His Met Glu Ser Glu Glu Leu Asn Gly Ala Tyr Lys
                165                 170                 175

gcc atc ccc gtt gcc cag gac ctg aac gcg cct tct gat tgg gac agc   576
Ala Ile Pro Val Ala Gln Asp Leu Asn Ala Pro Ser Asp Trp Asp Ser
            180                 185                 190

cgt ggg aag gac agt tat gaa acg agt cag ctg gat gac cag agt gct   624
Arg Gly Lys Asp Ser Tyr Glu Thr Ser Gln Leu Asp Asp Gln Ser Ala
        195                 200                 205

gaa acc cac agc cac aag cag tcc aga tta tat aag cgg aaa gct aat   672
Glu Thr His Ser His Lys Gln Ser Arg Leu Tyr Lys Arg Lys Ala Asn
    210                 215                 220

gat gag agc aat gag cat tcc gat gtg att gat agt cag gaa ctt tcc   720
Asp Glu Ser Asn Glu His Ser Asp Val Ile Asp Ser Gln Glu Leu Ser
225                 230                 235                 240

aaa gtc agc cgt gaa ttc cac agc cat gaa ttt cac agc cat gaa gat   768
Lys Val Ser Arg Glu Phe His Ser His Glu Phe His Ser His Glu Asp
                245                 250                 255
```

```
atg ctg gtt gta gac ccc aaa agt aag gaa gaa gat aaa cac ctg aaa   816
Met Leu Val Val Asp Pro Lys Ser Lys Glu Glu Asp Lys His Leu Lys
            260                 265                 270

ttt cgt att tct cat gaa tta gat agt gca tct tct gag gtc aat taa   864
Phe Arg Ile Ser His Glu Leu Asp Ser Ala Ser Ser Glu Val Asn
        275                 280                 285
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<213> Homo sapiens

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Met Arg Ile Ala Val Ile Cys Phe Cys Leu Leu Gly Ile Thr Cys Ala
  1               5                  10                  15

Ile Pro Val Lys Gln Ala Asp Ser Gly Ser Ser Glu Glu Lys Gln Asn
            20                  25                  30

Ala Val Ser Ser Glu Glu Thr Asn Asp Phe Lys Gln Glu Thr Leu Pro
        35                  40                  45

Ser Lys Ser Asn Glu Ser His Asp His Met Asp Asp Met Asp Asp Glu
    50                  55                  60

Asp Asp Asp Asp His Val Asp Ser Gln Asp Ser Ile Asp Ser Asn Asp
65                  70                  75                  80

Ser Asp Asp Val Asp Asp Thr Asp Asp Ser His Gln Ser Asp Glu Ser
                85                  90                  95

His His Ser Asp Glu Ser Asp Glu Leu Val Thr Asp Phe Pro Thr Asp
            100                 105                 110

Leu Pro Ala Thr Glu Val Phe Thr Pro Val Val Pro Thr Val Asp Thr
        115                 120                 125

Tyr Asp Gly Arg Gly Asp Ser Val Val Tyr Gly Leu Arg Ser Lys Ser
    130                 135                 140

Lys Lys Phe Arg Arg Pro Asp Ile Gln Tyr Pro Asp Ala Thr Asp Glu
145                 150                 155                 160

His Ile Thr Ser His Met Glu Ser Glu Glu Leu Asn Gly Ala Tyr Lys
                165                 170                 175

Ala Ile Pro Val Ala Gln Asp Leu Asn Ala Pro Ser Asp Trp Asp Ser
            180                 185                 190

Arg Gly Lys Asp Ser Tyr Glu Thr Ser Gln Leu Asp Asp Gln Ser Ala
        195                 200                 205

Glu Ala His Ser His Lys Gln Ser Arg Leu Tyr Lys Arg Lys Ala Asn
    210                 215                 220
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Asp Glu Ser Asn Glu His Ser Asp Val Ile Asp Ser Gln Glu Leu Ser
225                 230                 235                 240

Lys Val Ser Arg Glu Phe His Ser His Glu Phe His Ser His Glu Asp
                245                 250                 255

Met Leu Val Val Asp Pro Lys Ser Lys Glu Glu Asp Lys His Leu Lys
            260                 265                 270

Phe Arg Ile Ser His Glu Leu Asp Ser Ala Ser Ser Glu Val Asn
        275                 280                 285
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(74) Agents: **HANLEY, Elizabeth, A.** et al.; Lahive & Cockfield, LLP, 28 State Street, Boston, MA 02109 (US).

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(54) Title: METHODS AND COMPOSITIONS FOR MODULATING AN IMMUNE RESPONSE

(57) Abstract: The present invention features new approaches for modulating immune responses. In particular, the invention features methods for modulating type 1 immune responses in a subject using modulators of Eta-1(early T lymphocyte activation-1)/osteopontin. Examplary methods feature methods of treating infections, immune disorders and diseases, autoimmune disorders and diseases, various immunodeficiencies and cancer. Also provided are biosynthetic immunomodulatory molecules that include functional domains derived from Eta-1/osteopontin. Preferred biosynthetic immunomodulatory molecules include an IL-12 stimulatory domain derived from Eta-1/osteopontin or an IL-10 inhibitory domain derived from Eta-1/osteopontin. The immunomodulatory molecules of the present invention are capable of biasing an immune response in a subject towards a type 1 immune response. Accordingly, therapeutic uses are disclosed which are based on the biosynthetic immunomodulatory molecules of the present invention.

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C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 56405 A (WHITEHEAD INSTITUTE FOR BIOMEDICAL RESEARCH) 17 December 1998 (1998-12-17) page 23 -page 27 ---	1-13,23, 24,28, 29,32, 35,36, 41-43
X	YU X Q ET AL: "A functional role for osteopontin in experimental crescentic glomerulonephritis in the rat." PROCEEDINGS OF THE ASSOCIATION OF AMERICAN PHYSICIANS, (1998 JAN-FEB) 110 (1) 50-64. , XP000982503 the whole document --- -/--	1-13,23, 24,28, 29,32, 35,36, 41-43

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"&" document member of the same patent family

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European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Moreau, J

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	WEBER GEORG F ET AL: "The immunology of Eta-1/Osteopontin." CYTOKINE & GROWTH FACTOR REVIEWS, vol. 7, no. 3, 1996, pages 241-248, XP000982501 ISSN: 1359-6101 the whole document	1-70
P,X	ASHKAR SAMY ET AL: "Eta-1 (osteopontin): An early component of type-1 (cell-mediated) immunity." SCIENCE (WASHINGTON D C)., vol. 287, no. 5454, 4 February 2000 (2000-02-04), pages 860-864, XP002161278 ISSN: 0036-8075 the whole document	1-70

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WO 9856405 A	17-12-1998	NONE	

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(54) Title: METHODS AND COMPOSITIONS FOR MODULATING AN IMMUNE RESPONSE

(57) Abstract: The present invention features new approaches for modulating immune responses. In particular, the invention features methods for modulating type 1 immune responses in a subject using modulators of Eta-1(early T lymphocyte activation-1)/osteopontin. Examplary methods feature methods of treating infections, immune disorders and diseases, autoimmune disorders and diseases, various immunodeficiencies and cancer. Also provided are biosynthetic immunomodulatory molecules that include functional domains derived from Eta-1/osteopontin. Preferred biosynthetic immunomodulatory molecules include an IL-12 stimulatory domain derived from Eta-1/osteopontin or an IL-10 inhibitory domain derived from Eta-1/osteopontin. The immunomodulatory molecules of the present invention are capable of biasing an immune response in a subject towards a type 1 immune response. Accordingly, therapeutic uses are disclosed which are based on the biosynthetic immunomodulatory molecules of the present invention.

METHODS AND COMPOSITIONS FOR MODULATING AN IMMUNE RESPONSE

Related Applications

This application claims the benefit of previously filed U.S. Provisional Application Serial No. 60/129,772, filed April 15, 1999, the content of which is hereby incorporated by reference. This invention was made with government support from the National Institutes of Health. Accordingly, the government may have certain rights in the invention.

Background of the Invention

Efficient development of inflammatory responses and protection against most infectious pathogens depends, in part, on monocytes as the final effector cells. The participation of monocytes in inflammation entails emigration of these cells from peripheral blood into infected tissues, where they produce cytokines that regulate diverse processes including anti-microbial activity, cell growth, differentiation and wound healing (Singer *et al.* (1995) *J. Clin. Invest.* 95:2178-2186. In acute reactions, monocytes may be attracted by neutrophils whereas, in delayed responses, they act in a neutrophil-independent manner. Secretion of T-cell cytokines plays a pivotal role in recruitment of monocytes to sites of infection and activation of these emigrant cells to express bacteriocidal activity. The mechanism of this process bears intensely on wound healing and delayed-type immune responses but its molecular basis is not understood.

An important component of this T-cell dependent response is a protein known as Eta-1 (for early T lymphocyte activation-1)/osteopontin, which mediates macrophage chemotaxis *in vitro* (Weber *et al.* (1996) *Science* 271:509-512, recruits monocytes to inflammatory sites *in vivo* (Singh *et al.* (1990) *J. Exp. Med.* 171:1931-1942) and regulates immunological resistance to several intracellular pathogens (Patarca *et al.* (1989) *J. Exp. Med.* 170:145-161; Lampe *et al.* (1991) *J. Immunol.* 147:2902-2906. Inbred mouse strains that carry an allele of Eta-1/osteopontin which allows high level expression in activated T-cells are resistant to lethal effects of infection by the intracellular parasite *Rickettsia tsutsugamushi* while inbred strains carrying a low expression allele do not develop a population of bacteriocidal monocytic migrants at the

area of infection and succumb to systemic bacteremia (Patarca *et al.* (1990) *Immunol. Rev.* (116:1-16). Eta-1/osteopontin expression has also been linked to granuloma formation, where it may regulate the chronic cellular response associated with tuberculosis infection and silicosis (Nau *et al.* (1997) *Proc. Natl. Acad. Sci. USA* 94:6414-6419. Moreover, in experimental glomerulonephritis, neutralizing antibodies to osteopontin greatly diminish the influx of macrophages and T-cells and reduce kidney damage (Yu *et al.* (1998) *Proc. Am. Assoc. Physicians* 110:50-64). While Eta-1/ostepontin has been implicated in at least certain immunological reactions, its precise role in the immune system has not previously been established. Moreover, Eta-1 is a multifunctional protein having diverse biological roles including , but not limited to, bone resorption, neoplastic transformation, atheromatous plaque formation, dystrophic calcification of inflamed and/or damaged tissues and resistance to certain bacterial infections. (See *e.g.*, Oldberg *et al.* (1986) *Proc. Natl. Acad. Sci. USA* 83:8819; Ross *et al.* (1993) *J. Biol. Chem.* 268:9901; Giachelli *et al.* (1995) *Ann. NY Acad. Sci.* 760:109; Senger *et al.* (1983) *Nature* 302:714; and Srivatsa *at al.* (1997) *J. Clin. Invest.* 99:996). With regards in particular, to understanding the role of Eta-1/osteopontin in immunity, there exists a need to understand the precise role that Eta-1/osteopontin plays in regulating immune responses in order to develop new approaches to treating immune disorders and diseases.

Summary of the Invention

The present invention establishes that Eta-1/osteopontin is a critical regulator of type-1 (cell-mediated) immunity and that this molecule includes a domain that promotes the production of the type 1 cytokine IL-12 and a domain that inhibits the production of the type 2 cytokine IL-10. Thus, the invention provides for the use of Eta-1/osteopontin modulatory agents (i.e. agents that stimulate or inhibit Eta-1/osteopontin activity) to bias an immune response either toward type 1 or type 2 immunity, depending on the clinical situation. The present invention identifies Eta-1/osteopontin as a critical cytokine in type 1 immune responses, in particular, in delayed type hypersensitivity responses. The invention defines Eta-1/osteopontin as a multifunctional molecule which acts as both a stimulator of IL-12 secretion by macrophages and an inhibitor of IL-10 expression. As such, Eta-1/osteopontin serves to bias an organism's cytokine pattern

towards that of a type 1 immune response. In particular, induction of IL-12 and inhibition of IL-10 reflect differential engagement of macrophage receptors: a phosphorylation-dependent interaction between the N-terminal portion of Eta-1/osteopontin and its integrin receptor on macrophages leads to IL-12 expression, while a phosphorylation-independent interaction of a C-terminal domain of Eta-1/osteopontin with CD44 mediates IL-10 inhibition. Moreover, cleavage of Eta-1/osteopontin by thrombin results in a C-terminal fragment of the cytokine which interacts with CD44 and induces macrophage chemotaxis, while engagement of integrin receptors by a non-overlapping N-terminal fragment leads to macrophage spreading and activation.

Based, at least in part, on a detailed understanding of the role this multifunctional cytokine plays in type 1 immune responses, the present invention features novel approaches to modulating immune responses, in particular, in potentiating type 1 immune responses. The invention further features new methods of treating disorders that may benefit from either a type 1 or type 2 immune response. More specifically, the identification of Eta-1/osteopontin as a critical regulator of type 1 immunity allows for selective manipulation of T cell subsets in a variety of clinical situations using the modulatory methods of the invention. The stimulatory methods of the invention (*i.e.*, methods that use an Eta-1/osteopontin stimulatory agent) upregulate the production of the Th1-associated cytokine IL-12 and/or dowregulate the production of the Th2-associated cytokine IL-10, with concomitant promotion of a type 1 immune responses and downregulation of type 2 immune responses. These stimulatory methods that promote a type 1 response can be used, for example, in the treatment of infections (e.g., bacterial, viral), cancer, allergy, burn-associated sepsis and immunodeficiency disorders. In contrast, the inhibitory methods of the invention (*i.e.*, methods that use an Eta-1/osteopontin inhibitory agent) downregulate the production of the Th1-associated cytokine IL-12 and/or upregulate the production of the Th2-associated cytokine IL-10, with concomitant downregulation of a type 1 immune responses and promotion of type 2 immune responses. These inhibitory methods that promote type 2 responses can be used, for example, in the treatment of autoimmune disorders, transplant rejection, granulomatous disorders, herpes simplex keratitis and bacterial arthritis.

Moreover, based on a detailed understanding of the functional domains of Eta-1/osteopontin, the present invention provides biosynthetic molecules which mimic distinct functions of Eta-1/osteopontin for use in a variety of therapeutic applications, in particular, in wound healing, enhancement of the immune response and in treatment of granulomatous disease. In particular, the biosynthetic molecules of the present invention are useful in biasing an immune response towards a delayed type hypersensitivity response, *i.e.*, towards type 1 immunity. A preferred IL-12 stimulatory domain of Eta-1/osteopontin comprises amino acids 71-168 of SEQ ID NO: 2. A preferred IL-10 inhibitory domain of Eta-1/osteopontin comprises amino acids 169-266 of SEQ ID NO: 2. Other features and advantages of the invention will be apparent from the following detailed description, and from the claims.

Brief Description of the Drawings

Figure 1A-C demonstrates gramuloma formation in normal, cytokine-deficient and Eta-1/osteopontin-deficient mice. *Figure 1A* depicts the data as the mean number of granulomas per high-power field (HPF) (X200 magnification), mean number of cells per granuloma, and as the product of these two indices, termed "granuloma burden". (Error bars indicate 1 SEM.). *Figure 1B* depicts an analysis of surface antigens expressed by cells within granulomas in the indicated mouse strains. *Figure 1C* depicts cytokine expression by cells from lymph nodes draining the site of granulomas. Data are representative of three independent experiments.

Figure 2A-E demonstrates HSV-1-specific delayed-type hypersensitivity (DHT) reactions in normal and Eta-1/opn$^{-/-}$ mice. *Figure 2A* depicts footpad swelling in Eta-1/opn$^{-/-}$ vs. Eta-1/opn$^{+/+}$ mice inoculated with HSV-1. The right (control, □) and left (HSV-1, ■) footpads of each mouse were measured 24h later using a micrometer. Each data point represents the mean and standard error of three mice/group. *Figure 2B* depicts inhibition of the HSV-1 DHT response in Eta-1/opn$^{+/+}$ mice by acute depletion of Eta-1/opn. *Figure 2C* depicts HSK in Eta-1/opn$^{-/-}$ (open circles) vs. Eta-1/opn$^{+/+}$ (closed circles) mice inoculated with HSV-1. *Figure 2D* depicts HSK in BALB/cBγJ (open circles), Eta-1/opn$^{-/-}$ (closed circles), Eta-1/opn$^{+/+}$ (open squares), and CB-17 (closes squares) mice inoculated with HSV-1. *Figure2E* depicts the cytokine response after

restimulation of draining lymph node cells (from mice 15 days after HSV-1 infection in vivo) or splenic macrophages.

Figure 3A-D demonstrates the differential regulation of macrophage IL-12 and IL-10 responses by purified Eta-1/opn. *Figure 3A* depicts the dose-dependent induction of IL-12 secretion, but not IL-10 secretion, from macrophages by Eta-1/opn. Assays were performed in quadruplets and each data point represents the mean and standard error of two independent experiments. *Figure 3B* depicts selective IL-12 secretion as stimulated by Eta-1/opn as compared to LPS and/or IL-4 which stimulate both IL-12 and IL-10 secretion. Assays were performed in quadruplets and each data point represents the mean and standard error of two independent experiments. *Figure 3C* demonstrates that the inhibitory effects of Eta-1/opn on IL-4-induced IL-10 production by macrophage are unaffected by the presence of anti-IL-12 antibody. Assays were performed in quadruplets and each point represents the mean and standard error of two independent experiments. *Figure 3D* depicts the inhibitory effect of Eta-1 on LPS-activated macrophage IL-10 production. Assays were performed in quadruplets, and each point represents the mean and standard error (error bars) or two independent experiments.

Figure 4 depicts the attachment and spreading of MH-S macrophages on phosphorylated Eta-1/opn, Eta-1/opn, Eta-1/opn-N-terminal fragment, and Eta-1/opn-C-terminal fragment. *Figure 4A* depicts the attachment and spreading of MH-S cells on phosphorylated Eta-1/opn, Eta-1/opn, Eta-1/opn-NT and Eta-1/opn-CT in the presence or absence of the peptide GRGDS (SEQ ID NO:11). *Figure 4B* depicts the correlation between PI-3K activation and spreading of cells on Eta-1/opn, dephosphorylated Eta-1/opn, C- or N-terminal fragment, or NK10 fragment.

Figure 5A-C demonstrates that induction of IL-12 and inhibition of IL-10 occur *via* distinct receptors on macrophages. *Figure 5A* demonstrates that secretion of IL-12 by macrophages is mediated by a 10 kD (NK10) peptide derived from the N-terminal fragment of Eta-1/opn (NT) and is inhibited by a blocking anti-integrin β_3 antibody but is unaffected by antibody to CD44. *Figure 5B* demonstrates that Eta-1/opn-dependent inhibition of IL-4-induced Il-10 production is reversed by anti-CD44 but not by anti-integrin antibodies. *Figure 5C* demonstrates that macrophages from $CD44^{-/-}$ mice are resistant to OEta-1/opn inhibition of the IL-10 response as compared to control mice in

which Eta-1/opn inhibits IL-4 induced IL-10 production. In all panels, mean values and standard errors from at least four data points are shown.

Figure 6A-B demonstrates that that phosphorylation of Eta-1/opn is necessary for engagement of integrin receptors leading to IL-12 production but not for ligation of CD44 leading to IL-10 inhibition by macrophages. *Figure 6A* depicts IL-12 secretion resulting from phosphorylated vs. unphosphorylated Eta-1/opn. *Figure 6B* demonstrates that dephosphorylation of native Eta-1/opn results in loss of IL-12 inducing activity, while phosphorylation of (inactive) recombinant Eta-1/opn restores this function.

Figure 7 is a bar graph demonstrating that ligation of integrin receptors on macrophages (*e.g.*, *via* Eta-1/osteopontin, recombinant phosphorylated Eta-1/osteopontin, N terminal fragment or NK10) causes predominantly IL-12, TGFβ, and TNFα secretion.

Figure 8 is a bar graph demonstrating that ligation of integrin receptors on macrophages causes predominantly IL-12, TGFβ, and TNFα secretion and depicts the effect of various inhibitors (*e.g.*, wortmanin, genestein, chelerythine, pertussis toxin, cytochalasin D, and N-(2-metylpiperazyl)-5-isoquinolinesulfoamide(H-7)) on the cytokine secretion profile. The data are represented as cytokine concentration in media harvested from appropriately treated cells.

Figure 9 is a bar graph representing the data of Figure 8 as a fold-induction of cytokine secreted.

Figure 10 is a schematic diagram of a biosynthetic immunomodulatory molecule of the present invention, termed "immunomodulin-2".

Figure 11 is a schematic diagram of a biosynthetic immunomodulatory molecule of the present invention, termed "immunomodulin-1".

Figure 12 is a bar graph depicting the effect of the biosynthetic immunomodulatory molecules immunomodulin-1 and immunomodulin-2 on IL-12 and IL-10 secretion by macrophages. Data are represented as cytokine concentration in media harvested from appropriately treated cells.

Figure 13 is a bar graph depicting the effect of the biosynthetic immunomodulatory molecules immunomodulin-1 and immunomodulin-2 on IL-12 and IL-10 secretion by macrophages stimulated with IL-4 or LPS. Data are represented as cytokine concentration in media harvested from appropriately treated cells.

Figure 14 is a bar graph depicting the effect of immunomodulin-2 administration in an *in vivo* model of allergy.

Detailed Description of the Invention

The present invention is based, at least in part, on the elucidation of a new role for Eta-1/osteopontin in regulating immune responses, in particular, as a modulator of type-1 immunity. It has been discovered that Eta-1/osteopontin plays a dual role in activation of, for example, the type-1 cytokine IL-12, as well as in the inhibition of the type-2 cytokine IL-10. As such, Eta-1/osteopontin is capable of biasing an immune response in favor of a type-1 response, or a cellular immune response, as compared with a type-2 response, or humoral response. It has been further discovered that the IL-12 stimulatory and IL-10 inhibitory functions of Eta-1/osteopontin can be localized to a specific domains of the naturally-occurring protein. Identification of these biologically active domains of Eta-1/osteopontin has led to the development of new approaches to and therapeutics useful for the treatment of various immune response-related diseases and disorders. Moreover, the role of Eta-1/osteopontin in processes including monocyte recruitment, adhesion and activation (*i.e.*, cytokine secretion) has been analyzed in detail and new mechanisms for performing such functions have been disclosed.

In one aspect, the invention features methods of modulating immune responses, in particular, methods of modulating type-1 immune responses in a subject *or* patient (e.g., a human subject *or patient*) which involve administering to the subject *or* patient an Eta-1/osteopontin modulator such that the immune response (e.g., the type-1 immune response) is modulated. In one embodiment, the Eta-1/osteopontin modulator stimulates Eta-1/osteopontin activity and the type-1 immune response is potentiated. In another embodiment, the Eta-1/osteopontin modulator inhibits Eta-1/osteopontin activity and the type-1 immune response is downregulated. In another embodiment, the Eta-1/osteopontin modulator is administered in a therapeutically effective amount. In another embodiment, the method also includes monitoring the type-1 response in the

subject (*e.g.*, determining the level of a detectable indicator of the type-1 response) and/or comparing the level of the detectable indicator to a control.

In another embodiment, the invention features methods of potentiating type-1 immune responses in a subject or patient that include selecting a patient or subject suffering from a disease or disorder that would benefit from a potentiated type-1 immune response (*e.g.*, selecting an individual patient or subject from the human population) and administering to that patient an Eta-1/osteopontin stimulatory modulator such that the type-1 immune response is potentiated. In a preferred embodiment, the disease or disorder that would benefit from a potentiated type-1 immune response is at least one of the following: (1) burn-associated sepsis, (2) bacterial infection, (3) viral infection, (4) cancer, (5) immunodeficiency disorders, (6) AIDS, (7) bone marrow transplant-related immunodeficiency, (7) chemotherapy-related immunodeficiency and (7) allergy.

In another embodiment, the invention features methods of downregulating type-1 immune responses in a subject or patient that include selecting a patient or subject suffering from a disorder that would benefit from a downregulated type-1 immune response (*e.g.*, selecting an individual patient or subject from the human population) and administering to the patient or subject an Eta-1/osteopontin inhibitory modulator such that the type-1 immune response is downregulated. In a preferred embodiment, the disease or disorder that would benefit from a downregulated type-1 immune response is at least one of the following: (1) bacterial arthritis, (2) granulomatous disorder, (3) herpes simplex keratitis, and (4) autoimmune diseases.

In yet another embodiment, the present invention features methods for enhancing production of a type-1 immune response-associated cytokine (*e.g*, interleukin-2 (IL-2), interleukin-12 (IL-12) and/or interferon-γ (IFN-γ)) by an immune cell (*e.g.*, a human immune cell) that include contacting the cell with an Eta-1/osteopontin stimulatory modulator such that production of the cytokine is enhanced. In yet another embodiment, the invention features methods for downregulating production of a type-2 immune response-associated cytokine (*e.g.*, interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6), and/or interleukin-10 (IL-10)) by an immune cell that include contacting (*e.g.*, *in vivo* or *in vitro*) the cell with an Eta-1/osteopontin inhibitory modulator such that production of the cytokine is downregulated. Exemplary

immune cells include macrophages, dendritic cells, T cells, B cells, monocytes and/or neutrophils. In yet another embodiment, the invention features methods for stimulating interleukin-12 (IL-12) production by macrophages that include contacting the macrophages with an Eta-1/osteopontin stimulatory modulator such that production of IL-12 is stimulated. In yet another embodiment, the invention features a method for inhibiting interleukin-10 (IL-10) production by macrophages that includes contacting the macrophages with an Eta-1/osteopontin stimulatory modulator such that production of IL-10 is inhibited.

The present invention also features methods for potentiating type-1 immune responses in a subject or patient that include culturing immune effector cells isolated from the subject or patient in the presence of an Eta-1/osteopontin stimulatory modulator and administering the cultured cells to the subject such that the type-1 immune response in the subject is potentiated. Also featured are modified tumor cells, for example, irradiated tumor cells transduced with Eta-1/osteopontin and such modified tumor cells further transduced with GMCSF.

Preferred Eta-1/osteopontin modulators of the present invention include, but are not limited to, isolated Eta-1/osteopontin polypeptides and biologically active fragments thereof, isolated nucleic acid molecules that encodes Eta-1/osteopontin polypeptides and that encode biologically active fragments thereof. In one embodiment, the Eta-1/osteopontin modulator is an Eta-1/osteopontin polypeptide at least 90% identical to a polypeptide having the amino acid sequence of SEQ ID NO:2. In another embodiment, the Eta-1/osteopontin modulator is an Eta-1/osteopontin polypeptide having the amino acid sequence of SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6.

In another embodiment, the Eta-1/osteopontin modulator is an isolated nucleic acid molecule at least 90% identical to a nucleic acid molecule having the nucleotide sequence of SEQ ID NO:1. In yet another embodiment, the Eta-1/osteopontin modulator is an isolated nucleic acid molecule having the nucleotide sequence of SEQ ID NO:1, SEQ ID NO:3 or SEQ ID NO:5.

In another embodiment, the Eta-1/osteopontin modulator is a biologically active fragment of Eta-1/osteopontin, or a nucleic acid molecule encoding such a biologically active fragment. Preferred biologically active fragments include IL-12 stimulatory domains and/or IL-10 inhibitory domains of Eta-1/osteopontin. In one

embodiment, an IL-12 stimulatory domain includes an amino acid sequence between 65 and 160 amino acids in length and is at least 90% identical to amino acids 71 to 168 of SEQ ID NO:2. In another embodiment, an IL-10 inhibitory domain includes an amino acid sequence between 65 and 160 amino acids in length and is at least 90% identical to amino acids 169-266 of SEQ ID NO:2.

Additional preferred Eta-1/osteopontin modulators of the present invention include compounds that specifically bind to Eta-1/osteopontin polypeptides, compounds that specifically binds to Eta-1/osteopontin target molecules, compounds that specifically modulate the activity of Eta-1/osteopontin polypeptides and compounds that specifically modulate the activity of Eta-1/osteopontin target molecules. In one embodiment, the Eta-1/osteopontin modulator is an antibody that specifically binds to Eta-1/osteopontin. In another embodiment, the Eta-1/osteopontin modulator is an antibody that specifically binds an Eta-1/osteopontin target molecule (*e.g.*, an antibody that specifically binds to an integrin or a CD44 molecule).

In yet another embodiment, the Eta-1/osteopontin modulator is a biosynthetic immunomodulatory molecule. Preferred biosynthetic immunomodulatory molecules include an IL-12 stimulatory component (*e.g.*, an IL-12 stimulatory component derived from Eta-1/osteopontin) and a biomodular component, forming a molecule which modulates an immune response. For example, an IL-12 stimulatory component can be an Eta-1/osteopontin-derived polypeptide (*e.g.*, a polypeptide that has an amino acid sequence between 65 and 160 amino acid residues in length and is at least 90% identical to amino acids 71 to 168 of SEQ ID NO:2).

Additional preferred biosynthetic immunomodulatory molecules include an IL-10 inhibitory component (*e.g.*, an IL-10 inhibitory component derived from Eta-1/osteopontin) and a biomodular component, forming a molecule which modulates an immune response. For example, an IL-10 inhibitory component can be an Eta-1/osteopontin-derived polypeptide (*e.g.*, a polypeptide that has between 65 and 160 amino acid residues in length and is at least 90% identical to amino acids 169 to 266 of SEQ ID NO:2). Exemplary biomodular component include signal peptides, calcium/apatite binding domains and/or heparin binding domains. Additional preferred biosynthetic immunomodulatory molecules include at least two biomodular components.

A preferred biosynthetic immunomodulatory molecule includes [Picture claim for Immunomodulin-2] (*e.g.*, the biosynthetic immunomodulatory molecule comprising the amino acid sequence of SEQ ID NO:8 and/or the molecule encoded by nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO:7). Another preferred biosynthetic immunomodulatory molecule includes an IL-10 inhibitory component, a signal peptide, a calcium/apatite binding domain and a heparin binding domain (*e.g.*, the biosynthetic immunomodulatory molecule comprising the amino acid sequence of SEQ ID NO:10 and/or the molecule encoded by nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO:9).

Preferred biosynthetic immunomodulatory molecules of the present invention are useful for modulating an immune response (*e.g.*, in a subject or patient, for example, a human subject or patient) for example, in modulating cytokine secretion, regulation of chemotaxis, regulation of hapotaxis, and regulation of cell spreading. Also featured are isolated nucleic acid molecules that encode the biosynthetic immunomodulatory molecules of the present invention, expression vectors that include such nucleic acid molecules, and host cell including such vectors. The present invention also features methods of producing biosynthetic immunomodulatory molecule that include culturing such host cell under conditions such that the immunomodulatory molecule is produced. Pharmaceutical composition that include the biosynthetic immunomodulatory molecules of the present invention are also featured.

The present invention also features method of modulating an immune response in a cell that include contacting the cell with a featured biosynthetic immunomodulatory molecule such that an immune response is modulated. In a preferred embodiment the cell is present within a subject or patient and the immunomodulatory molecule is administered to the subject.

In order that the present invention may be more readily understood, certain terms are first defined herein.

The term "Eta-1/osteopontin" refers to a protein known in the art and referred to herein interchangeably as "early T lymphocyte activation-1", "Eta-1", "osteopontin", "opn" and "Eta-1/opn". Eta-1/osteopontin was originally identified in bone

and is now also known to be secreted T cells early during their activation by a variety of stimuli. Eta-1/osteopontin is a noncollagenous adhesive matrix protein normally found in bone and at epithelial cell surfaces. Eta-1/osteopontin contains an arginine-glycine-aspartate (RGD)-binding motif common to many extracellular matrix proteins. Eta-1/osteopontin also contains a thrombin cleavage site, cleavage of which alters the proteins adhesive properties. Eta-1 has at least two cellular receptors including integrin and CD44 (Weber *et al.* (1996) *Science* 271:509-512). As described herein, Eta-1 has muliple biological functions. In particular, Eta-1/osteopontin can function as an immune response modulator. (See *e.g.*, U.S. Patent No. 5,049,659 and WO 98/08379). A preferred biological function of Eta-1, as described herein, is in potentiating a type 1 immune response. For a detailed review of the structure and biological functions of Eta-1/osteopontin, see *e.g.*, Denhardt and Guo (1993) 7:1475-1482 and Patarca *et al.* (1993) *Crit. Revs. Imunol.* 13:225-246, and the referenced cites therein.

The term "immune response" includes any response associated with immunity including, but not limited to, increases or decreases in cytokine expression, production or secretion (*e.g.*, IL-12, IL-10, TGFβ or TNFα expression, production or secretion), cytotoxicity, immune cell migration, antibody production and/or immune cellular responses. The phrase "modulating an immune response" or "modulation of an immune response" includes upregulation, potentiating, stimulating, enhancing or increasing an immune response, as defined herein. For example, an immune response can be upregulated, enhanced, stimulated or increased directly by use of a modulator of the present invention (*e.g.*, a stimulatory modulator). Alternatively, a modulator can be used to "potentiate" an immune response, for example, by enhancing, stimulating or increasing immune responsiveness to a stimulatory modulator. The phrase "modulating an immune response" or "modulation of an immune response" also includes downregulation, inhibition or decreasing an immune response as defined herein. Immune responses in a subject or patient can be further characterized as being either type-1 or type-2 immune responses.

A "type-1 immune response", also referred to herein as a "type-1 response" or a "T helper type 1 (Th1) response" includes a response by $CD4^+$ T cells that is characterized by the expression, production or secretion of one or more type-1 cytokines and that is associated with delayed type hypersensitivity responses. The

phrase "type-1 cytokine" includes a cytokine that is preferentially or exclusively expressed, produced or secreted by a Th1 cell, that favors development of Th1 cells and/or that potentiates, enhances or otherwise mediates delayed type hypersensitivity reactions. Preferred type-1 cytokines include, but are not limited to, interleukin-2 (IL-2), interleukin-12 (IL-12), interferon-γ (IFN-γ) and tumor necrosis factor-β (TNF-β).

A "type-2 immune response", also referred to herein as a "type-2 response or a "T helper type 2 (Th2) response" refers to a response by $CD4^+$ T cells that is characterized by the production of one or more type-2 cytokines and that is associated with humoral or antibody-mediated immunity (*e.g.*, efficient B cell, "help" provided by Th2 cells, for example, leading to enhanced IgG1 and/or IgE production). The phrase "type-2 cytokine" includes a cytokine that is preferentially or exclusively expressed, produced or secreted by a Th2 cell, that favors development of Th2 cells and/or that potentiates, enhances or otherwise mediates antibody production by B lymphocytes. Preferred type-2 cytokines include, but are not limited to, interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6), interleukin-10 (IL-10) and interleukin-13 (IL-13).

Cytokine expression, secretion or production modulates or further enhances or upregulates an immune response, for example, a type-1 of type-2 immune response. For example, it is known that cytokines play a dominant role in controlling the differentiation of T helper precursors (Th0) to either the Th1 or Th2 lineage. Type-1 cytokines, such as IFN-γ, can enhance the development of Th1 cells and inhibit the development of Th2 cells, whereas type-2 cytokines, such as IL-4 and IL-10, can enhance the development of Th2 cells and inhibit the development of Th1 cells. Thus, cytokines can reciprocally regulate the development and/or progression of either a type-1 or a type-2 response.

Cytokine expression, secretion or production can also be an indicator of an immune response, for example, an indicator of a type-1 or type-2 immune response.

For example, a "cytokine profile" can be indicative of a type-1 or type-2 immune response. The term "cytokine profile" includes expression, production or secretion of at least one cytokine associated with a particular type of immune response (*e.g.*, a type-1 or type-2 immune response) and/or includes diminished or reduced expression, production or secretion of at least one cytokine associated with a mutually

exclusive type of immune response (*e.g.*, a type-2 or type-1 immune response, respectively). For example, a type-1 cytokine profile can include enhanced or increased expression, production or secretion of at least one of interleukin-2 (IL-2), interleukin-12 (IL-12), interferon-γ (IFN-γ) and tumor necrosis factor-β (TNF-β) and/or can include reduced or decreased expression, production or secretion of at least one of interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6) and interleukin-10 (IL-10). Likewise, a type-2 cytokine profile can include expression, production or secretion of at least one of interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6) and interleukin-10 (IL-10) and/or can include reduced or decreased expression, production or secretion of at least one of interleukin-2 (IL-2), interleukin-12 (IL-12), interferon-γ (IFN-γ) and tumor necrosis factor-β (TNF-β).

The phrase "type-1 immunity" includes immunity characterized predominantly by type-1 immune responses (*e.g.*, delayed type hypersensitivity, macrophage activation and or cellular cytotoxicity), by expression, production or secretion of at least one type-1 cytokine and/or expression of a type-1 immunity cytokine profile. The phrase "type-2 immunity" includes immunity characterized predominantly by type-2 immune responses (*e.g.*, B cell help, IgG1 and/or IgE production, eosinophil activation, mast cell stimulation and/or macrophage deactivation), by expression, production or secretion of at least one type-2 cytokine and/or expression of a type-2 immunity cytokine profile.

The course of certain disease states is influenced by whether a predominant type-1 or type-2 response is mounted. For example, in experimental leishmania infections in mice, animals that are resistant to infection mount predominantly a type-1 immune response, whereas animals that are susceptible to progressive infection mount predominantly a type-2 immune response (Heinzel *et al.* (1989) *J. Exp. Med.* 169:59-72; and Locksley and Scott (1992) *Immunoparasitology Today* 1:A58-A61). In murine schistosomiasis, a switch from type-1 to type-2 immunity is observed coincident with the release of eggs into the tissues by female parasites and is associated with a worsening of the disease condition (Pearce *et al.* (1991) *J. Exp. Med.* 173:159-166; Grzych *et al.* (1991) *J. Immunol.* 141:1322-1327; and Kullberg *et al.* (1992) *J. Immunol.* 148:3264-3270). Many human diseases, including chronic infections (such as with human immunodeficiency virus (HIV) or tuberculosis) and

certain metastatic carcinomas, also are characterized by a type-1 to type-2 switch. (see *e.g.*, Shearer and Clerici (1992) *Prog. Chem. Immunol.* 54:21-43; Clerici and Shearer (1993) *Immunol. Today* 14:107-111; Yamamura *et al.* (1993) *J. Clin. Invest.* 91:1005-1010; Pisa *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:7708-7712; Fauci (1988) *Science* 239:617-623). Furthermore, certain autoimmune diseases have been shown to be associated with a predominant type-1 response. For example, patients with rheumatoid arthritis have predominantly Th1 cells in synovial tissue (Simon *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:8562-8566) and experimental autoimmune encephalomyelitis (EAE) can be induced by autoreactive Th1 cells (Kuchroo *et al.* (1993) *J. Immunol.* 151:4371-4381).

The phrase "potentiating or potentiation of a type-1 or type-2 immune response" includes upregulation, stimulation or enhancement of a type-1 or type-2 response, respectively (*e.g.*, commitment of T helper precursors to either a Th1 or Th2 lineage, further differentiation of cells to either the Th1 or Th2 phenotype and/or continued function of Th1 or Th2 cells during an ongoing immune response). For a review of Th1 and Th2 subsets see, for example, Seder and Paul (1994) *Ann. Rev. Immunol.* 12:635-673.

The phrase "potentiating or potentiation of a type-1 immune response" also includes downregulation or inhibition of a type-2 immune response. The phrase "potentiating or potentiation of a type-2 immune response" also includes downregulation or inhibition of a type-1 immune response.

The term "immunomodulatory molecule", used interchangeably herein with the term "immunomodulatory "agent" includes a molecule or agent which has a modulatory or regulatory activity which is normally associated with an immune response in an organism, for example, higher animals and humans. An activity (*e.g.*, a biological or functional activity) associated with an immune response can be any activity associated with resistance of the organism to infection with microorganisms, response to infection or response to disease. The term "activity", "biological activity" or "functional activity", refers to an activity exerted by a molecule of the invention (*e.g.*, a immunomodulatory molecule, for example, a protein, polypeptide, fragment, nucleic acid molecule, antibody, biosynthetic immunomodulatory molecule, or the like) as

determined *in vivo*, or *in vitro*, according to standard techniques and/or methods such as those described in the Examples.

The term "immune cell" includes cells of the immune system which are capable of expressing, producing or secreting cytokines that regulate an immune response, for example a type-1 or type-2 immune response. Preferred immune cells include human immune cells. Exemplary preferred immune cells include, but are not limited to, macrophages, dendritic cells, T cells, B cells and neutrophils. Immune cells are also referred to herein as "immune effector cells". The term "macrophage" includes all cells within the macrophage lineage, including monocytes, circulating macrophages, tissue macrophages, activated macrophages, and the like, from mammals (*e.g.*, from humans). The term "T cell" (*i.e.*, T lymphocyte) is intended to include all cells within the T cell lineage, including thymocytes, immature T cells, mature T cells and the like, from mammals (*e.g.*, from humans).

The phrase "contacting" (*e.g.*, contacting a cell, for example, with an agent or modulator) is intended to include incubating the agent and the cell together *in vitro* (*e.g.*, adding the agent or modulator to cells in culture) or alternatively, administering the agent or modulator to a subject or patient such that the agent or modulator is capable or contacting the cells of the subject or patient *in vivo*. "Administering" an agent or modulator includes any routine means known in the art or described herein of providing a subject or patient with an agent or modulator. "Coadministering" agents includes administering a first and second agent or modulator, for example, sequentially or coincidentally. In addition to administering agents and/or modulators (*e.g.*, immunomodulatory molecules), certain aspects of the present invention feature administering cells to a subject or patient. For example, cells of a patient (*e.g.*, immune cells or immune effector cells) can be isolated from a subject, contacted with an agent or modulator *in vitro* (*e.g.*, culturing the cells in the presence of the agent or modulator), and administered or readministered to the subject or patient. Routine means can be utilized for isolating immune cells, for example, isolating and/or separating plasma from a subject or patient, isolating bone marrow from a patient or subject, as well as for administering or readministering cells, for example, plasmaphoresis or bone marrow transplants.

The term "subject" includes a living animal, preferably a human subject.

The term "patient" includes a subject, preferably a human subject, in need of treatment (*e.g.*, treatment according to the methodologies of the present invention), potentially in need of treatment, presently undergoing treatment, having or suffering from a disease or disorder which would benefit from at least one methodology of the present invention. Preferably a "patient" is a human patient.

Exemplary diseases and/or disorders from which a patient, as defined herein, may be at risk for, have or be suffering from include but are not limited to burn-associated sepsis, infectious diseases or disorders (*e.g.*, bacterial infection, viral infection, HIV and tuberculosis) cancer, immunodeficiency disorders, AIDS, bone marrow transplant-related immunodeficiency, chemotherapy-related immunodeficiency, allergy, bacterial arthritis, granulomatous disorder, herpes simplex keratitis, autoimmune disease, and various forms of glomerulonephritis including, but not limited to, rhematoid arthritis and multiple sclerosis. Diseases and disorders are to be given their accepted, art-recognized definitions, for example, as set forth in The Physicians Desk Reference.

The phrase "monitoring and immune response", for example, "monitoring a type-1 immune response" includes monitoring the ability of an agent or modulator of the invention to enhance, potentiate, stimulate, upregulate or downregulate or inhibit an immune response, for example, a type-1 or type-2 immune response. In one embodiment, monitoring a type-1 or type-2 response includes determining the level of a detectable indicator of the type-1 or type-2 response. Preferred detectable indicators include cytokines associated with a particular response, cytokine profiles associated with a particular response and/or phenotypic responses. Exemplary detectable indicators of a type-1 response include expression, production or secretion to type-1 cytokines, type-1 cytokine profiles, as well as any other type-1 phenotypic response, as described herein. Exemplary detectable indicators of a type-2 response include expression, production or secretion to type-2 cytokines, type-2 cytokine profiles, as well as any other type-2 phenotypic response, as described herein. In another embodiment, monitoring an immune response further comprises comparing the detectable indicator to a control (*e.g.*, a control profile or control phenotype, for example, the profile or phenotype of the subject or patient prior to treatment or at a previous stage of treatment with an agent or modulator, the profile or phenotype) of a

normal or average subject, or an expected or target profile or phenotype (*e.g.*, a theoretical, desired or predicted profile or phenotype).

Various aspects of the invention are described in further detail in the following subsections.

I. Immunomodulatory Agents

In the immunomodulatory methods of the invention, for example, type-1 immunomodulatory methods, an Eta-1/osteopontin modulator is administered to a subject (*e.g.*, a human subject) or a cell (*e.g.*, a human immune cell) is contacted with the modulator such that an immune response, for example, a type-1 immune response is modulated. In one embodiment, the Eta-1/osteopontin modulator is a "stimulatory agent" (*e.g.*, an agent or modulator that stimulates Eta-1/osteopontin activity), which enhances, potentiated, increases or upregulates a type-1 immune response in a cell or subject. Preferred "stimulatory agents" or "stimulatory modulators" include isolated Eta-1/osteopontin proteins or polypeptides and biologically active fragments thereof, isolated nucleic acid molecules encoding such Eta-1/osteopontin proteins or polypeptides and biologically active fragments thereof, biosynthetic immunomodulatory molecules, Eta-1 peptides, peptidomimetics and small molecule agonists (*e.g.*, Eta-1 peptides, peptidomimetics and small molecule agonists capable of specifically binding to an Eta-1/osteopontin receptor, for example, integrin or CD44, and/or upregulating the activity of the Eta-1/osteopontin receptor as described in further detail below. In another embodiment, the Eta-1/osteopontin modulator is an "inhibitory agent" (*e.g.*, an agent or modulator that inhibits Eta-1/osteopontin activity), which decreases or downregulates a type-1 immune response in a cell or subject. Preferred "inhibitory agents" or "inhibitory modulators" include antisense Eta-1/osteopontin nucleic acid molecules, Eta-1/osteopontin antibodies and/or Eta-1/osteopontin receptor antibodies (*e.g.* compounds capable of specifically binding to an Eta-1/osteopontin receptor, for example, integrin or CD44, and/or downregulating the activity of the Eta-1/osteopontin receptor), as described in further detail below. Additional preferred modulatory agents modulate selected activities of Eta-1/osteopontin, for example, modulate activities resulting from ligation of CD44 and/or integrin by Eta-1/osteopontin. Particularly

preferred modulatory agents modulate immune responses specific for Eta-1/osteopontin interacting with CD44 and/or integrin.

A. Isolated Eta-1/Osteopontin Proteins, Biologically-active Fragments Thereof and

One aspect of the invention pertains to isolated Eta-1/osteopontin proteins and biologically active portions thereof. Eta-1/osteopontin proteins can be isolated from cells or tissue sources by an appropriate purification scheme using standard protein purification techniques, can produced by recombinant DNA techniques or can be synthesized chemically using standard peptide synthesis techniques. Biologically active portions of Eta-1/osteopontin polypeptides can be further generated by enzymatic digestion of full-length Eta-1/osteopontin polypeptides, can be produced by recombinant DNA techniques or can be synthesized chemically using standard peptide synthesis techniques.

An "isolated" or "purified" Eta-1/osteopontin protein or biologically active portion thereof is substantially free of cellular material or other contaminating proteins from the cell or tissue source from which the Eta-1/osteopontin is derived, or substantially free from chemical precursors or other chemicals when chemically synthesized. The language "substantially free of cellular material" includes preparations of Eta-1/osteopontin in which the protein is separated from cellular components of the cells from which it is isolated or recombinantly produced. The language "substantially free of cellular material" includes preparations in which the recombinant molecule is separated from cellular components of the cells from which it is isolated or recombinantly produced. In one embodiment, the language "substantially free of cellular material" includes preparations having less than about 30% (by dry weight) of contaminating cellular material, more preferably less than about 20% of contaminating material, still more preferably less than about 10% of contaminating material, and most preferably less than about 5% contaminating material. When Eta-1/osteopontin is recombinantly produced, it is also preferably substantially free of culture medium, *i.e.*, culture medium represents less than about 20%, more preferably less than about 10%, and most preferably less than about 5% of the volume of the preparation.

The language "substantially free of chemical precursors or other chemicals" includes preparations in which the chemically synthesized molecule is separated from chemical precursors or other chemicals which are involved in the synthesis of the molecule. In one embodiment, the language "substantially free of chemical precursors or other chemicals" includes preparations having less than about 30% (by dry weight) of chemical precursors or contaminating chemicals, more preferably less than about 20% chemical precursors or contaminating chemicals, still more preferably less than about 10% chemical precursors or contaminating chemicals, and most preferably less than about 5% chemical precursors or contaminating chemicals.

In a preferred embodiment, an Eta-1/osteopontin protein for use in the present invention is a human Eta-1/osteopontin. For example, any of the Eta-1/osteopontin proteins set forth as SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6 are suitable for use in the immunomodulatory methods of the present invention (*e.g.*, Eta-1/osteopontin protein-a, Eta-1/osteopontin protein-b or Eta-1/osteopontin protein-c, respectively). Also suitable for use in the immunomodulatory methods of the present invention are Eta-1/osteopontin homologues or variants which vary at the amino acid sequence level when compared, for example, to the Eta-1/osteopontin proteins set forth as SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6 but which retain the biological activity of the Eta-1/osteopontin proteins set forth as SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6. For example, Eta-1/osteopontin homologues or variants having 85-90%, 90-95%, 96%, 97%, 98%, 99% or more sequence identity to the Eta-1/osteopontin proteins set forth as SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6 but which retain biological activity are suitable for use in the immunomodulatory methods of the present invention. Eta-1/osteopontin homologues or variants can have amino acid substitutions (particularly conservative amino acid substitutions) at "non-essential" amino acid residues in the sequence of SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6. A "non-essential" amino acid residue is a residue that can be altered from the sequence set forth in SEQ ID NO:2, SEQ ID NO:4 or SEQ ID NO:6 without altering the biological activity, whereas an "essential" amino acid residue is required for biological activity. For example, amino acid residues that are conserved among proteins or domains of proteins from different species are predicted to be particularly unamenable to alteration.

Alternatively, Eta-1/osteopontin homologues or variants can have a conservative amino acid substitutions at one or more predicted essential or non-essential amino acid residues. A "conservative amino acid substitution" is one in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (*e.g.*, lysine, arginine, histidine), acidic side chains (*e.g.*, aspartic acid, glutamic acid), uncharged polar side chains (*e.g.*, glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine), nonpolar side chains (*e.g.*, alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan), beta-branched side chains (*e.g.*, threonine, valine, isoleucine) and aromatic side chains (*e.g.*, tyrosine, phenylalanine, tryptophan, histidine). Thus, a predicted essential or nonessential amino acid residue is preferably replaced with another amino acid residue from the same side chain family.

Also suitable for use in the immunomodulatory methods of the present invention are Eta-1/osteopontin homologues or variants which are encoded by nucleic acid molecules comprising the nucleotide sequences of SEQ ID NO:1, SEQ ID NO:3 or SEQ ID NO:5, as well as Eta-1/osteopontin homologues or variants encoded by nucleic acid molecules having 85-90%, 90-95%, 96%, 97%, 98%, 99% or more sequence identity to the Eta-1/osteopontin nucleotide sequences of SEQ ID NO:1, SEQ ID NO:3 or SEQ ID NO:5 and/or isolated nucleic acid molecules which hybridize under stringent hybridization conditions to the Eta-1/osteopontin nucleotide sequences of SEQ ID NO:1, SEQ ID NO:3 or SEQ ID NO:5. As used herein, the term "hybridizes under stringent conditions" is intended to describe conditions for hybridization and washing under which nucleotide sequences at least 90% homologous to each other typically remain hybridized to each other. Such stringent conditions are known to those skilled in the art and can be found in *Current Protocols in Molecular Biology*, John Wiley & Sons, N.Y. (1992). A preferred, non-limiting example of stringent hybridization conditions are hybridization in 6X sodium chloride/sodium citrate (SSC) at about 45°C, followed by one or more washes in 0.2 X SSC, 0.1% SDS at 50-65°C.

Biologically active portions of Eta-1/osteopontin include fragments or portions sufficiently homologous to Eta-1/osteopontin, *e.g.*, the amino acid sequence shown in SEQ ID NO:2, which include less amino acids than the full length polypeptide,

and exhibit at least one activity of full-length Eta-1/osteopontin. Typically, biologically active portions comprise a domain with at least one activity of the full-length polypeptide. A biologically active portion can be a polypeptide which is, for example, 50-100, 100-150, 150-200, 200-250, 250-300 or more amino acids in length.

In one embodiment, a biologically active portion of Eta-1/osteopontin comprises an IL-12 stimulatory domain of Eta-1/osteopontin. As used herein, an IL-12 stimulatory domain is a domain of Eta-1/osteopontin capable of ligating integrin (*e.g.*, αvβ3 integrin) such that IL-12 production is stimulated by the cell. In a preferred embodiment, an IL-12 stimulatory domain is incapable of ligating CD44 expressed on the cell. In one embodiment, an IL-12 stimulatory domain is about 50-60, 60-70, 70-80, 80-90 or 90-100 amino acid residues in length. In another embodiment, an IL-12 stimulatory domain is of a size sufficient to induce IL-12 production by a cell but includes insufficient amino acid residues to inhibit IL-10 production by the cell. A particularly preferred IL-12 stimulatory domain includes the residues of the fragment NK10 described herein.

In another embodiment, a biologically active portion of Eta-1/osteopontin comprises an IL-10 inhibitory domain of Eta-1/osteopontin. As used herein, an IL-10 inhibitory domain is a domain of Eta-1/osteopontin capable of ligating CD44 such that IL-10 production is inhibited by the cell. In a preferred embodiment, an IL-10 inhibitory domain is incapable of ligating integrin (*e.g.*, αvβ3 integrin) expressed on the cell. In one embodiment, an IL-10 inhibitory domain is about 50-60, 60-70, 70-80, 80-90 or 90-100 amino acid residues in length. In another embodiment, an IL-10 inhibitory domain is of a size sufficient to inhibit IL-10 production by a cell but includes insufficient amino acid residues to stimulate IL-12 production by the cell. A particularly preferred IL-10 inhibitory domain includes about amino acid residues 169-200, 169-220, 169-240, 169-260, or 169-280 of SEQ ID NO:2.

To determine the percent homology of two amino acid sequences or of two nucleic acid sequences, the sequences are aligned for optimal comparison purposes (*e.g.*, gaps can be introduced in the sequence of a first amino acid or nucleic acid sequence for optimal alignment with a second amino or nucleic acid sequence). The amino acid residues or nucleotides at corresponding amino acid positions or nucleotide positions are then compared. When a position in the first sequence is occupied by the

same amino acid residue or nucleotide as the corresponding position in the second sequence, then the molecules are homologous at that position (*i.e.*, as used herein amino acid or nucleic acid "homology" is equivalent to amino acid or nucleic acid "identity"). The percent homology between the two sequences is a function of the number of identical positions shared by the sequences (*i.e.*, % homology = # of identical positions/total # of positions x 100). The determination of percent homology between two sequences can be accomplished using a mathematical algorithm. A preferred, non-limiting example of a mathematical algorithm utilized for the comparison of two sequences is the algorithm of Karlin and Altschul (1990) *Proc. Natl. Acad. Sci. USA* 87:2264-68, modified as in Karlin and Altschul (1993) *Proc. Natl. Acad. Sci. USA* 90:5873-77. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul *et al.* (1990) *J. Mol. Biol.* 215:403-10. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12 to obtain nucleotide sequences homologous to the nucleic acid molecule sequences of the invention. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3 to obtain amino acid sequences homologous to the protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul *et al.* (1997) *Nucleic Acids Research* 25(17):3389-3402. When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) can be used. See http://www.ncbi.nlm.nih.gov. Another preferred, non-limiting example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Myers and Miller (1988) *Comput Appl Biosci.* 4:11-17. Such an algorithm is incorporated into the ALIGN program available, for example, at the GENESTREAM network server, IGH Montpellier, FRANCE (http://vega.igh.cnrs.fr) or at the ISREC server (http://www.ch.embnet.org). When utilizing the ALIGN program for comparing amino acid sequences, a PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used.

The invention also provides chimeric or fusion proteins, for example, recombinant chimeric or fusion proteins designed to facilitate the purification of Eta-1/osteopontin (*e.g.*, GST-fusion proteins or HA-tagged fusion proteins). Also provided are chimeric or fusion proteins (*e.g.*, Eta-1/osteopontin containing a heterologous signal

sequence at its N-terminus) to enhance expression and/or secretion recombinant Eta-1/osteopontin. Chimeric or fusion proteins of the invention are produced by standard recombinant DNA techniques as described. for example. in *Current Protocols in Molecular Biology*, eds. Ausubel et al. John Wiley & Sons: 1992). Moreover. many expression vectors are commercially available that already encode a fusion moiety (*e.g.*, a GST polypeptide). An Eta-1/osteopontin-encoding nucleic acid, as described herein. can be cloned into such an expression vector such that the fusion moiety is linked in-frame to the Eta-1/osteopontin protein.

Also featured are Eta-1/osteopontin proteins and biologically active portions which are incorporated into pharmaceutical compositions as described herein.

B. Isolated Nucleic Acid Molecules, Vectors, Host Cells

Another aspect of the invention pertains to isolated nucleic acid molecules that encode Eta-1/osteopontin proteins or portions or biologically active fragments thereof. The term "nucleic acid molecule" includes DNA molecules (e.g., cDNA or genomic DNA) and RNA molecules (e.g., mRNA) and analogs of the DNA or RNA generated using nucleotide analogs. The nucleic acid molecule can be single-stranded or double-stranded, but preferably is double-stranded DNA.

An "isolated" nucleic acid molecule is one which is separated from other nucleic acid molecules which are present in the natural source of the nucleic acid. Preferably, an "isolated" nucleic acid is free of sequences which naturally flank the nucleic acid (i.e., sequences located at the 5` and 3' ends of the nucleic acid) in the genomic DNA of the organism from which the nucleic acid is derived. For example, in various embodiments, the isolated nucleic acid molecule can contain less than about 5 kb, 4kb, 3kb, 2kb, 1 kb, 0.5 kb or 0.1 kb of nucleotide sequences which naturally flank the nucleic acid molecule in genomic DNA of the cell from which the nucleic acid is derived. Moreover, an "isolated" nucleic acid molecule, such as a cDNA molecule, can be substantially free of other cellular material, or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized.

In another preferred embodiment, an isolated nucleic acid molecule of the invention comprises a nucleotide sequence shown in SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5 or a portion thereof. Also included within the scope of the invention are isolated nucleic acid molecules which are complementary to the nucleotide sequences shown in SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5 or a portion thereof. Also included within the scope of the present invention are isolated nucleic acid molecules which hybridize (*e.g.*, under stringent hybridization conditions) to a complement of the nucleotide sequences shown in SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5 or a portion thereof, thereby forming a stable duplex. Also included within the scope of the present invention are isolated nucleic acid molecules having 80-85%, 90-95%, 96%, 97%, 98%, 99% or more homology or identity to the nucleotide sequences shown in SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5 or a portion thereof. Also included within the scope of the present invention are isolated nucleic acid molecules which are antisense to the nucleic acid molecules shown in SEQ ID NO:1, SEQ ID NO:3, SEQ ID NO:5. The invention further encompasses nucleic acid molecules that differ from the nucleotide sequence shown in SEQ ID NO:1, SEQ ID NO:3 or SEQ ID NO:5 yet due to degeneracy of the genetic code encode the same molecules as those encoded by the nucleotide sequence shown in SEQ ID NO:1, SEQ ID NO:3 or SEQ ID NO:5.

A nucleic acid molecule of the invention, or portion thereof, can be amplified using cDNA, mRNA or alternatively, genomic DNA, as a template and appropriate oligonucleotide primers according to standard PCR amplification techniques. The nucleic acid so amplified can be cloned into an appropriate vector and characterized by DNA sequence analysis. Furthermore, oligonucleotides (*e.g.*, probes and/or primers) and antisense nucleic acid molecules can be prepared by standard synthetic techniques, *e.g.*, using an automated DNA synthesizer. Oligonucleotides for use in the present invention typically comprises substantially purified oligonucleotide. The oligonucleotide typically comprises a region of nucleotide sequence that hybridizes under stringent conditions to at least about 12, preferably about 25, more preferably about 40, 50 or 75 consecutive nucleotides of the nucleotide sequence of SEQ ID NO:1, SEQ ID NO:3 or SEQ ID NO:5 of or of the complement of the nucleotide sequence of SEQ ID NO:1, SEQ ID NO:3 or SEQ ID NO:5. Also included within the scope of the present invention are oligonucleotides at least 15, 30, 50, 100, 250 or 500 nucleotides in

length which hybridizes under stringent conditions to the nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO:1, SEQ ID NO:3 or SEQ ID NO:5.

A nucleic acid fragment encoding a "biologically active" portion of an Eta-1/osteopontin molecule of the present invention can be prepared by isolating a portion of SEQ ID NO:1, SEQ ID NO:3 or SEQ ID NO:5 which encodes a polypeptide having a biological activity of the naturally-occurring protein from which the portion was derived, expressing the encoded portion of the naturally-occurring protein (*e.g.*, by recombinant expression *in vitro*) and assessing the activity of the encoded portion of the naturally-occurring protein. As used herein, a "naturally-occurring" nucleic acid molecule or protein molecule refers to a molecule having a nucleotide or amino acid sequence that occurs in nature (*e.g.*, a nucleic acid molecule that encodes a natural protein).

Another aspect of the invention pertains to vectors, preferably expression vectors, containing a nucleic acid molecule of the present invention. As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional DNA segments can be ligated. Another type of vector is a viral vector, wherein additional DNA segments can be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (*e.g.*, bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (*e.g.*, non-episomal mammalian vectors) are integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors are capable of directing the expression of genes to which they are operatively linked. Such vectors are referred to herein as "expression vectors". In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids. In the present specification, "plasmid" and "vector" can be used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors, such as viral vectors (*e.g.*, replication defective retroviruses, adenoviruses and adeno-associated viruses), which serve equivalent functions.

The recombinant expression vectors of the invention comprise a nucleic acid of the invention in a form suitable for expression of the nucleic acid in a host cell, which means that the recombinant expression vectors include one or more regulatory sequences, selected on the basis of the host cells to be used for expression, which is operatively linked to the nucleic acid sequence to be expressed. Within a recombinant expression vector, "operably linked" means that the nucleotide sequence of interest is linked to the regulatory sequence(s) in a manner which allows for expression of the nucleotide sequence (e.g., in an *in vitro* transcription/translation system or in a host cell when the vector is introduced into the host cell). The term "regulatory sequence" includes promoters, enhancers and other expression control elements (e.g., polyadenylation signals). Such regulatory sequences are described, for example, in Goeddel; *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990). Regulatory sequences include those which direct constitutive expression of a nucleotide sequence in many types of host cell and those which direct expression of the nucleotide sequence only in certain host cells (e.g., tissue-specific regulatory sequences). It will be appreciated by those skilled in the art that the design of the expression vector can depend on such factors as the choice of the host cell to be transformed, the level of expression of protein desired, etc. The expression vectors of the invention can be introduced into host cells to thereby produce proteins or peptides, including fusion proteins or peptides, encoded by nucleic acids as described herein.

The recombinant expression vectors of the invention can be designed for expression in prokaryotic or eukaryotic cells. For example, recombinant proteins can be expressed in bacterial cells such as *E. coli*, insect cells (using baculovirus expression vectors) yeast cells or mammalian cells. Suitable host cells are discussed further in Goeddel, *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, CA (1990). Alternatively, the recombinant expression vector can be transcribed and translated *in vitro*, for example using T7 promoter regulatory sequences and T7 polymerase.

Expression of proteins in prokaryotes is most often carried out in *E. coli* with vectors containing constitutive or inducible promotors directing the expression of either fusion or non-fusion proteins. Fusion vectors add a number of amino acids to a protein encoded therein, usually to the amino terminus of the recombinant protein. Such

fusion vectors typically serve three purposes: 1) to increase expression of recombinant protein; 2) to increase the solubility of the recombinant protein; and 3) to aid in the purification of the recombinant protein by acting as a ligand in affinity purification. Often, in fusion expression vectors, a proteolytic cleavage site is introduced at the junction of the fusion moiety and the recombinant protein to enable separation of the recombinant protein from the fusion moiety subsequent to purification of the fusion protein. Such enzymes, and their cognate recognition sequences, include Factor Xa, thrombin and enterokinase. Typical fusion expression vectors include pGEX (Pharmacia Biotech Inc; Smith, D.B. and Johnson, K.S. (1988) *Gene* 67:31-40), pMAL (New England Biolabs, Beverly, MA) and pRIT5 (Pharmacia, Piscataway, NJ) which fuse glutathione S-transferase (GST), maltose E binding protein, or protein A, respectively, to the target recombinant protein.

Examples of suitable inducible non-fusion *E. coli* expression vectors include pTrc (Amann et al., (1988) *Gene* 69:301-315) and pET 11d (Studier et al., *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, California (1990) 60-89). Target gene expression from the pTrc vector relies on host RNA polymerase transcription from a hybrid trp-lac fusion promoter. Target gene expression from the pET 11d vector relies on transcription from a T7 gn10-lac fusion promoter mediated by a coexpressed viral RNA polymerase (T7 gn1). This viral polymerase is supplied by host strains BL21(DE3) or HMS174(DE3) from a resident λ prophage harboring a T7 gn1 gene under the transcriptional control of the lacUV 5 promoter.

One strategy to maximize recombinant protein expression in *E. coli* is to express the protein in a host bacteria with an impaired capacity to proteolytically cleave the recombinant protein (Gottesman, S., *Gene Expression Technology: Methods in Enzymology* 185, Academic Press, San Diego, California (1990) 119-128). Another strategy is to alter the nucleic acid sequence of the nucleic acid to be inserted into an expression vector so that the individual codons for each amino acid are those preferentially utilized in *E. coli* (Wada et al., (1992) *Nucleic Acids Res.* 20:2111-2118). Such alteration of nucleic acid sequences of the invention can be carried out by standard DNA synthesis techniques.

In another embodiment, the expression vector is a yeast expression vector. Examples of vectors for expression in yeast *S. cerivisae* include pYepSec1 (Baldari, et al., (1987) *Embo J.* 6:229-234), pMFa (Kurjan and Herskowitz, (1982) *Cell* 30:933-943), pJRY88 (Schultz et al., (1987) *Gene* 54:113-123), pYES2 (Invitrogen Corporation, San Diego, CA), and picZ (InVitrogen Corp, San Diego, CA).

Alternatively, recombinant proteins can be expressed in insect cells using baculovirus expression vectors. Baculovirus vectors available for expression of proteins in cultured insect cells (e.g., Sf 9 cells) include the pAc series (Smith et al. (1983) *Mol. Cell Biol.* 3:2156-2165) and the pVL series (Lucklow and Summers (1989) *Virology* 170:31-39).

In yet another embodiment, a nucleic acid of the invention is expressed in mammalian cells using a mammalian expression vector. Examples of mammalian expression vectors include pCDM8 (Seed, B. (1987) *Nature* 329:840) and pMT2PC (Kaufman et al. (1987) *EMBO J.* 6:187-195). When used in mammalian cells, the expression vector's control functions are often provided by viral regulatory elements. For example, commonly used promoters are derived from polyoma, Adenovirus 2, cytomegalovirus and Simian Virus 40. For other suitable expression systems for both prokaryotic and eukaryotic cells see chapters 16 and 17 of Sambrook, J., Fritsh, E. F., and Maniatis, T. *Molecular Cloning: A Laboratory Manual. 2nd, ed., Cold Spring Harbor Laboratory,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.

In another embodiment, the recombinant mammalian expression vector is capable of directing expression of the nucleic acid preferentially in a particular cell type (*e.g.*, tissue-specific regulatory elements are used to express the nucleic acid). Tissue-specific regulatory elements are known in the art. Non-limiting examples of suitable tissue-specific promoters include the albumin promoter (liver-specific; Pinkert *et al.* (1987) *Genes Dev.* 1:268-277), lymphoid-specific promoters (Calame and Eaton (1988) *Adv. Immunol.* 43:235-275), in particular promoters of T cell receptors (Winoto and Baltimore (1989) *EMBO J.* 8:729-733) and immunoglobulins (Banerji *et al.* (1983) *Cell* 33:729-740; Queen and Baltimore (1983) *Cell* 33:741-748), neuron-specific promoters (e.g., the neurofilament promoter; Byrne and Ruddle (1989) *PNAS* 86:5473-5477), pancreas-specific promoters (Edlund *et al.* (1985) *Science* 230:912-916), and mammary

gland-specific promoters (*e.g.*, milk whey promoter; U.S. Patent No. 4,873,316 and European Application Publication No. 264,166). Developmentally-regulated promoters are also encompassed, for example the murine hox promoters (Kessel and Gruss (1990) *Science* 249:374-379) and the α-fetoprotein promoter (Campes and Tilghman (1989) *Genes Dev.* 3:537-546).

The invention further provides a recombinant expression vector comprising a DNA molecule of the invention cloned into the expression vector in an antisense orientation. That is, the DNA molecule is operatively linked to a regulatory sequence in a manner which allows for expression (by transcription of the DNA molecule) of an RNA molecule which is antisense to an mRNA corresponding to a nucleic acid molecule of the present invention. Regulatory sequences operatively linked to a nucleic acid cloned in the antisense orientation can be chosen which direct the continuous expression of the antisense RNA molecule in a variety of cell types, for instance viral promoters and/or enhancers, or regulatory sequences can be chosen which direct constitutive, tissue specific or cell type specific expression of antisense RNA. The antisense expression vector can be in the form of a recombinant plasmid, phagemid or attenuated virus in which antisense nucleic acids are produced under the control of a high efficiency regulatory region, the activity of which can be determined by the cell type into which the vector is introduced. For a discussion of the regulation of gene expression using antisense genes see Weintraub, H. et al., Antisense RNA as a molecular tool for genetic analysis, *Reviews - Trends in Genetics*, Vol. 1(1) 1986.

Another aspect of the invention pertains to host cells into which a recombinant expression vector of the invention has been introduced. The terms "host cell" and "recombinant host cell" are used interchangeably herein. It is understood that such terms refer not only to the particular subject cell but to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

A host cell can be any prokaryotic or eukaryotic cell. For example, Immunomodulin protein can be expressed in bacterial cells such as *E. coli*, insect cells, yeast or mammalian cells (such as Chinese hamster ovary cells (CHO) or COS cells). Other suitable host cells are known to those skilled in the art.

Vector DNA can be introduced into prokaryotic or eukaryotic cells via conventional transformation or transfection techniques. As used herein, the terms "transformation" and "transfection" are intended to refer to a variety of art-recognized techniques for introducing foreign nucleic acid (e.g., DNA) into a host cell, including calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, or electroporation. Suitable methods for transforming or transfecting host cells can be found in Sambrook, et al. (*Molecular Cloning: A Laboratory Manual. 2nd, ed., Cold Spring Harbor Laboratory,* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989), and other laboratory manuals.

For stable transfection of mammalian cells, it is known that, depending upon the expression vector and transfection technique used, only a small fraction of cells may integrate the foreign DNA into their genome. In order to identify and select these integrants, a gene that encodes a selectable marker (*e.g.*, resistance to antibiotics) is generally introduced into the host cells along with the gene of interest. Preferred selectable markers include those which confer resistance to drugs, such as G418, hygromycin and methotrexate. Nucleic acid encoding a selectable marker can be introduced into a host cell on the same vector as that encoding recombinant proteins or can be introduced on a separate vector. Cells stably transfected with the introduced nucleic acid can be identified by drug selection (*e.g.*, cells that have incorporated the selectable marker gene will survive, while the other cells die).

A host cell of the invention, such as a prokaryotic or eukaryotic host cell in culture, can be used to produce (*i.e.*, express) recombinant protein. Accordingly, the invention further provides methods for producing recombinant protein using the host cells of the invention. In one embodiment, the method comprises culturing the host cell of invention (into which a recombinant expression vector encoding recombinant protein has been introduced) in a suitable medium such that the recombinant protein is produced. In another embodiment, the method further comprises isolating the recombinant protein from the medium or the host cell.

C. Activating, Neutralizing and/or Blocking Antibodies

As described herein, preferred Eta-1 modulators are agents that are capable of modulating select Eta-1/osteopontin-mediated activities, in particular, select activities associated with potentiation of a type-1 immune response. Accordingly, in one embodiment, the invention features methods of modulating an immune response which include administering an Eta-1 antibody, for example, an antibody which specifically block or neutralizes the interaction of Eta-1/osteopontin with a cell surface receptor (*e.g.*, CD44 and/or integrin $\alpha v\beta 3$). In one embodiment, an antibody is specific for the N-terminal, *e.g.*, IL-12 stimulatory domain of Eta-1/osteopontin, as defined herein. In another embodiment, the antibody is specific for the C-terminal, *e.g.*, IL-10 inhibitory domain if Eta-1/osteopontin. In yet another embodiment, the antibody is specific for the RGD sequence of Eta-1/osteopontin (*e.g.*, the integrin binding domain). In a preferred embodiment, the antibody is LF123, as described herein. Also included within the scope of the present invention are fragments of such antibodies, *e.g.*, Fab' fragments, humanized antibodies, and the like, for use as therapeutic agents.

D. Peptides, Peptidomimetics and Small Molecule Modulators

The present invention also pertains to Eta-1/osteopontin peptides, Eta-1/osteopontin peptidomimetics and or small molecule modulators of Eta-1/osteopontin which function as either Eta-1/osteopontin agonists (mimetics) or as Eta-1/osteopontin antagonists. An Eta-1/osteopontin agonists can retain substantially the same, or a subset, of the biological activities of the naturally occurring form of Eta-1/osteopontin. An Eta-1/osteopontin antagonist can inhibit one or more of the activities of the naturally occurring form of Eta-1/osteopontin. Thus, specific biological effects can be elicited by treatment with an Eta-1/osteopontin agonist or antagonist of limited function. In one embodiment, treatment of a subject with an Eta-1/osteopontin agonist or antagonist having a subset of the biological activities of the naturally occurring form of the protein has fewer side effects in a subject relative to treatment with the full length Eta-1/osteopontin.

Eta-1/osteopontin agonist or antagonist can be identified by screening libraries of Eta-1 peptides, combinatorial libraries based on Eta-1 peptides or small molecule libraries for Eta-1 agonist or antagonist activity. In one embodiment, a variegated library is generated by combinatorial mutagenesis at the Eta-1/osteopontin nucleic acid level and is encoded by a variegated gene library. Variegated libraries of compounds can be produced by, for example, enzymatically ligating a mixture of synthetic oligonucleotides into gene sequences such that a degenerate set of potential Eta-1/osteopontin sequences is expressible as individual polypeptides, or alternatively, as a set of larger fusion proteins (e.g., for phage display) containing the set of Eta-1/osteopontin sequences therein. There are a variety of methods which can be used to produce libraries of potential Eta-1/osteopontin variants from a degenerate oligonucleotide sequence. Chemical synthesis of a degenerate gene sequence can be performed in an automatic DNA synthesizer, and the synthetic gene then ligated into an appropriate expression vector. Use of a degenerate set of genes allows for the provision, in one mixture, of all of the sequences encoding the desired set of potential Eta-1/osteopontin sequences. Methods for synthesizing degenerate oligonucleotides are known in the art (see, e.g., Narang, S.A. (1983) *Tetrahedron* 39:3; Itakura et al. (1984) *Annu. Rev. Biochem.* 53:323; Itakura et al. (1984) *Science* 198:1056; Ike et al. (1983) *Nucleic Acid Res.* 11:477.

In addition, libraries of fragments of Eta-1/osteopontin coding sequence can be used to generate a variegated population of Eta-1/osteopontin fragments for screening and subsequent selection of variants of Eta-1/osteopontin. In one embodiment, a library of coding sequence fragments can be generated by treating a double stranded PCR fragment of an Eta-1/osteopontin coding sequence with a nuclease under conditions wherein nicking occurs only about once per molecule, denaturing the double stranded DNA, renaturing the DNA to form double stranded DNA which can include sense/antisense pairs from different nicked products, removing single stranded portions from reformed duplexes by treatment with S1 nuclease, and ligating the resulting fragment library into an expression vector. By this method, an expression library can be derived which encodes N-terminal, C-terminal and internal fragments of various sizes of the Eta-1/osteopontin protein.

Additional exemplary methods for the synthesis of molecular libraries can be found in the art, for example in: DeWitt *et al.* (1993) *Proc. Natl. Acad. Sci. U.S.A.* 90:6909; Erb *et al.* (1994) *Proc. Natl. Acad. Sci. USA* 91:11422; Zuckermann *et al.* (1994). *J. Med. Chem.* 37:2678; Cho et al. (1993) *Science* 261:1303; Carrell *et al.* (1994) *Angew. Chem. Int. Ed. Engl.* 33:2059; Carell *et al.* (1994) *Angew. Chem. Int. Ed. Engl.* 33:2061; and in Gallop *et al.* (1994) *J. Med. Chem.* 37:1233.

Libraries of compounds may be presented in solution (e.g., Houghten (1992) *Biotechniques* 13:412-421), or on beads (Lam (1991) *Nature* 354:82-84), chips (Fodor (1993) *Nature* 364:555-556), bacteria (Ladner USP 5,223,409), spores (Ladner USP '409), plasmids (Cull *et al.* (1992) *Proc Natl Acad Sci USA* 89:1865-1869) or on phage (Scott and Smith (1990) *Science* 249:386-390); (Devlin (1990) *Science* 249:404-406); (Cwirla *et al.* (1990) *Proc. Natl. Acad. Sci.* 87:6378-6382); (Felici (1991) *J. Mol. Biol.* 222:301-310); (Ladner *supra.*).

Additional test compounds can be obtained using any of the numerous approaches in combinatorial library methods known in the art, including: biological libraries; spatially addressable parallel solid phase or solution phase libraries; synthetic library methods requiring deconvolution; the 'one-bead one-compound' library method; and synthetic library methods using affinity chromatography selection. The biological library approach is limited to peptide libraries, while the other four approaches are applicable to peptide, non-peptide oligomer or small molecule libraries of compounds (Lam, K.S. (1997) *Anticancer Drug Des.* 12:145).

A. Screening Assays:

Several techniques are known in the art for screening libraries (*e.g.*, combinatorial libraries or small molecule libraries) for compounds having a selected property. Such techniques are preferably adaptable for rapid screening of the libraries described herein. Particularly preferred techniques are those which are amenable to high through-put analysis.

For example, candidate or test compounds can be screened for their ability to modulate the interaction of Eta-1/osteopontin with a CD44 or integrin receptor. In one embodiment, an assay is a cell-based assay in which a cell which expresses CD44 or integrin on the cell surface is contacted with a test compound.

optionally in the presence of Eta-1/osteopontin, and the ability of the test compound to modulate the interaction of Eta-1/osteopontin with a CD44 or integrin receptor is determined. The cell can be of mammalian origin, for example, a macrophage. Determining the ability of the test compound to modulate the interaction of Eta-1/osteopontin with a CD44 or integrin receptor can be accomplished, for example, by coupling the test compound (or Eta-1/osteopontin) with a radioisotope or enzymatic label such that binding to CD44 or integrin can be determined by detecting the labeled compound in a complex. For example, test compounds can be labeled with ^{125}I, ^{35}S, ^{14}C, or ^{3}H, either directly or indirectly, and the radioisotope detected by direct counting of radioemmission or by scintillation counting. Alternatively, reagents can be enzymatically labeled with, for example, horseradish peroxidase, alkaline phosphatase, or luciferase, and the enzymatic label detected by determination of conversion of an appropriate substrate to product.

The ability of a test compound to modulate the interaction of Eta-1/osteopontin with a CD44 or integrin receptor can also be determined without the labeling of any of the interactants. For example, a microphysiometer can be used to detect the interaction of components without the labeling of either the test compound or the receptor. McConnell, H. M. *et al.* (1992) *Science* 257:1906-1912. Determining the ability to modulate the interaction of Eta-1/osteopontin with a CD44 or integrin receptor can also be accomplished by determining, for example, induction of a cellular second messenger (i.e. intracellular Ca^{2+}, diacylglycerol, IP_3, etc.), detecting the induction of a reporter gene, or detecting a cellular response, for example, a proliferative response or an inflammatory response.

In yet another embodiment, an assay of the present invention is a cell-free assay in which an Eta-1/osteopontin protein or biologically active portion thereof is contacted with a test compound and the ability of the test compound to bind to the Eta-1/osteopontin protein or biologically active portion thereof is determined. Binding of the test compound to the Eta-1/osteopontin protein can be determined either directly or indirectly as described above. Binding of the test compound to the Eta-1/osteopontin protein can also be accomplished using a technology such as real-time Biomolecular Interaction Analysis (BIA). Sjolander, S. and Urbaniczky, C. (1991) *Anal. Chem.* 63:2338-2345 and Szabo *et al.* (1995) *Curr. Opin. Struct. Biol.* 5:699-705. As used

herein, "BIA" is a technology for studying biospecific interactions in real time, without labeling any of the interactants (e.g., BIAcore™). Changes in the optical phenomenon of surface plasmon resonance (SPR) can be used as an indication of real-time reactions between biological molecules.

In a preferred embodiment, the assay includes contacting the Eta-1/osteopontin protein or biologically active portion thereof with a known ligand which binds Eta-1/osteopontin to form an assay mixture, contacting the assay mixture with a test compound, and determining the ability of the test compound to interact with an Eta-1/osteopontin protein, wherein determining the ability of the test compound to interact with an Eta-1/osteopontin protein comprises determining the ability of the test compound to preferentially bind to Eta-1/osteopontin or biologically active portion thereof as compared to the known ligand.

In another embodiment, the assay is a cell-free assay in which a CD44 receptor or integrin receptor is contacted with a test compound (and optionally with Eta-1/osteopontin) and the ability of the test compound to modulate (e.g., stimulate or inhibit) the resulting interaction is determined. Cell-free assays of the present invention are amenable to use of both soluble and/or membrane-bound forms of receptors. In the case of cell-free assays in which a receptor is used it may be desirable to utilize a solubilizing agent such that the membrane-bound form of the isolated protein is maintained in solution. Examples of such solubilizing agents include non-ionic detergents such as n-octylglucoside, n-dodecylglucoside, n-dodecylmaltoside, octanoyl-N-methylglucamide, decanoyl-N-methylglucamide, Triton® X-100, Triton® X-114, Thesit®, Isotridecypoly(ethylene glycol ether)$_n$, 3-[(3-cholamidopropyl)dimethylamminio]-1-propane sulfonate (CHAPS), 3-[(3-cholamidopropyl)dimethylamminio]-2-hydroxy-1-propane sulfonate (CHAPSO), or N-dodecyl=N,N-dimethyl-3-ammonio-1-propane sulfonate.

In more than one of the above assay methods, it may be desirable to immobilize at least one assay reagent to facilitate separation of complexed from uncomplexed forms of one or both of the reagents, as well as to accommodate automation of the assay, for example, glutathione-S-transferase/ fusion proteins can be adsorbed onto glutathione sepharose beads (Sigma Chemical, St. Louis, MO) or glutathione derivatized microtitre plates. Alternatively at least one reagent can be immobilized utilizing conjugation of biotin and

streptavidin. Alternatively, antibodies reactive at least one reagent can be derivatized to wells or plates to immobilize reagents.

Novel agents identified by the above-described screening assays can be tested in an appropriate animal model, for example, to determine the efficacy, toxicity, or side effects of treatment with such an agent. Alternatively, agent can be tested in at least one of the *in vitro* or *in situ* assays described herein.

II. Biosynthetic Immunomodulatory Molecules

Based on the discovery of an immunomodulatory function of Eta-1/osteopontin, and in particular, the discovery of the IL-12 stimulatory and IL-10 inhibitory domains of Eta-1/osteopontin, the present invention features biosynthetic molecules which are modeled after these key functional domains. The biosynthetic molecules are useful in regulating a variety of cellular processes as well as in modulating immune responses. In particular, the biosynthetic immunomodulatory molecules are useful in biasing an immune response from a type-1 to a type-2 immune response.

As used herein, the term "biosynthetic molecule" includes molecules which have a biological activity and which are built or synthesized by the combination or union of components or elements that are simpler than the biosynthesized molecule. A biosynthetic molecule of the present invention is made or built by the hand of man (including automated processes) and accordingly, is distinguishable from a naturally-occurring molecule which is results from a naturally-occurring biological process. Alternatively, an organism can be used to produce a biosynthetic molecule of the present invention, provided that at least at one step in the synthesis, there is the intervention of man.

Accordingly, in one embodiment, the present invention features biosynthetic immunomodulatory molecules which include an IL-12 stimulatory component and a biomodular component, forming a molecule which modulates an immune response. The term "IL-12 stimulatory component" includes a piece or constituent of a molecule (*e.g.*, a fragment of Eta-1/osteopontin) which is smaller than the molecule of which it is a part, which functions to stimulate, enhance, upregulate the expression, production and/or secretion of the cytokine, IL-12, from a cell. A molecule which includes an IL-12 stimulatory component, for example, is capable of causing a cell capable of

expressing, producing and/or secreting IL-12..to express and/or secrete more of the cytokine in the presence of the IL-12 stimulatory component than in the absence of the IL-12 stimulatory component.

In another embodiment, the present invention features biosynthetic immunomodulatory molecules which include an IL-10 inhibitory component and a biomodular component, forming a molecule which modulates an immune response. The term "IL-10 inhibitory component" includes a piece or constituent of a molecule which is smaller than the molecule of which it is a part, which functions to inhibit the expression and/or secretion of the cytokine, IL-10, from a cell. A molecule which includes an IL-10 inhibitory component, for example, is capable of causing a cell capable of expressing and/or secreting IL-10, to express and/or secrete less of the cytokine in the presence of the IL-10 inhibitory component than in the absence of the IL-10 inhibitory component.

In addition to the IL-12 stimulatory component or the IL-10 inhibitory component defined herein, the biosynthetic immunomodulatory molecules of the present invention can include a biomodular component. The term "biomodular component" includes a piece or constituent of a molecule which is smaller than the molecule of which it is a part, which has either a biological function which is distinct from that of the IL-12 stimulatory component, the IL-10 inhibitory component or has a biological structure which is distinct from that of the IL-12 stimulatory component or the IL-10 inhibitory component. A biomodular component is a piece or constituent that either is not found in a naturally-occurring molecule which includes an IL-12 stimulatory component or an IL-10 inhibitory component (*e.g.*, Eta-1/osteopontin) or is not found in the same proximal relation to an IL-12 stimulatory component or an IL-10 inhibitory component as it exists within a naturally-occurring molecule. In one embodiment, a biomodular component is a polypeptide. Polypeptide biomodular components of the present invention include, but are not limited to signal peptides, a calcium/apatite binding domains and a heparin binding domains.

The term "signal peptide" or "signal sequence" refers to a peptide containing about 20 amino acids which occurs at the N-terminus of secretory and integral membrane proteins and which contains a large number of hydrophobic amino acid residues. For example, a signal sequence contains at least about 14-28 amino acid

residues, preferably about 16-26 amino acid residues, more preferably about 18-24 amino acid residues, and more preferably about 20-22 amino acid residues, and has at least about 40-70%, preferably about 50-65%, and more preferably about 55-60% hydrophobic amino acid residues (e.g., Alanine, Valine, Leucine, Isoleucine, Phenylalanine, Tyrosine, Tryptophan, or Proline). Such a "signal sequence", also referred to in the art as a "signal peptide", serves to direct a protein containing such a sequence from the endoplasmic reticulum of a cell to the golgi apparatus and ultimately to a lipid bilayer (*e.g.*, for secretion).

The term "calcium/apatite binding domain" includes a domain which, when included within a protein, polypeptide, or biosynthetic molecule of the present invention, functions to bind calcium, bind metal ions, or bind apatite (*e.g.*, hydroxyapatite). A "calcium/apatite binding domain" can also be referred to as a "6-Asp" domain. Also preferred is a 6-His domain. 6-Asp and 6-His domains are particularly useful for purification of the biosynthetic molecules of the preset invention.

The term "heparin binding domain" includes a domain which, when included within a protein, polypeptide, or biosynthetic molecule of the present invention, functions to bind the protein, polypeptide, or biosynthetic molecule to heparin. A "heparin binding domain" further includes a domain which has within it at least one, preferably two, three, four, five, six, or more "heparin binding domain minimum repeating units". The term "heparin binding domain minimum repeating unit" includes the consensus motif basic residue - basic residue - any residue - basic residue. Preferably, a "heparin binding domain minimum repeating unit" has the sequence arginine – arginine – any residue – arginine. Also preferred are collagen binding domains. Heparin binding domains and/or collagen binding domains are particularly useful for stabilizing the biosynthetic molecules of the present invention, *e.g.*, for anchoring or adhering the molecules to ECM surrounding target cells of the invention.

Accordingly, a biosynthetic immunomodulatory molecule of the present invention is formed by the combination of at least an IL-12 stimulatory component or an IL-10 inhibitory domain and a biomodular component. The term "formed" or "forming" includes the bringing together of at least two components into a structural and/or functional association. For example, a recombinant nucleic acid molecule can be formed by the bringing together of at least two nucleic acid components. Alternatively,

a recombinant protein can be formed by the bringing together of at least two protein components. Moreover, a composition can be formed by the bringing together of at least two compositions.

In a preferred embodiment, the present invention features biosynthetic molecules which include an IL-10 inhibitory component which is derived from Eta-1/osteopontin. A component "derived from", for example, Eta-1/osteopontin, includes a component which has certain features which originate from Eta-1/osteopontin and are recognizable as such, but which is not identical to Eta-1/osteopontin. In one embodiment, an IL-10 inhibitory component is a polypeptide which is derived from Eta-1/osteopontin. Accordingly, the IL-10 inhibitory component has features of Eta-1/osteopontin (*e.g.*, functions to inhibit IL-10 secretion) but is not identical to osteopontin. In one embodiment, an IL-10 inhibitory component includes a polypeptide which has at least 50% identity to an IL-10 inhibitory domain of Eta-1/osteopontin. In yet another embodiment, an IL-10 inhibitory component is at least 55%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or more identical to an IL-10 inhibitory domain of Eta-1/osteopontin IL-10. In yet another embodiment, an IL-10 inhibitory component includes a polypeptide which has at least 80-85%, 85-90%, 90-95%, 96%, 97%, 98%, 99% or more identity to about amino acids 169-266 of SEQ ID NO:2 In another embodiment, an IL-10 inhibitory component includes a polypeptide which is at least 65-160 amino acids in length. In another embodiment, an IL-10 inhibitory component includes a polypeptide which is between 30-35, 40-45, 45-50, 50-55, 55-60, 60-65, 65-70, 70-75, 75-80, 80-85, 85-90 90-100 or more amino acids in length. In another embodiment, an IL-10 inhibitory component includes a polypeptide which is greater than 115 amino acids in length.

Another embodiment of the present invention features biosynthetic molecules which include an IL-10 inhibitory component having an amino acid sequence sufficiently homologous to the an IL-10 inhibitory domain of a protein having the amino acid sequence of Eta-1/osteopontin (*e.g.*, SEQ ID NO:2), as defined herein. In a preferred embodiment, an IL-10 inhibitory component retains an IL-10 inhibitory, preferably an IL-10 inhibitory activity of Eta-1/osteopontin. In another embodiment, a molecule has an immunomodulatory activity. In another embodiment, an IL-10 inhibitory component includes an amino acid sequence selected from the group

consisting of amino acids 71-180 of SEQ ID NO:4, amino acids 58-166 of SEQ ID NO:6, or amino acids 44-153 of SEQ ID NO:8.

The present invention further features isolated nucleic acid molecules which encode the biosynthetic immunomodulatory molecules of the present invention. In one embodiment, an isolated nucleic acid molecule of the present invention includes a nucleic acid sequence which encodes an IL-10 inhibitory domain. In another embodiment, an isolated nucleic acid molecule of the present invention includes a nucleic acid sequence which encodes a biomodulatory domain. In another embodiment, an isolated nucleic acid molecule of the present invention includes a nucleic acid sequence (SEQ ID NO:9) which encodes Immunomodulin-1 (SEQ ID NO:10).

A. Isolated Biosynthetic Molecules

"Isolated" or "purified" biosynthetic molecules are also features according to the present invention. "Isolated" or "purified" biosynthetic molecules are substantially free of cellular material or other contaminating proteins from the cell or tissue source from which the molecule is derived, or substantially free from chemical precursors or other chemicals when chemically synthesized. The phrases "substantially free of cellular material" and "substantially free of chemical precursors or other chemicals" are as defined herein for isolated Eta-1/proteins or polypeptides.

Also featured are isolated nucleic acid molecules encoding the biosynthetic molecules of the present invention, vectors including such nucleic acid molecules, as well as host cells into which such vectors have been incorporated, as defined herein. Also featured are methods of making the biosynthetic molecules of the present invention, as described herein for making Eta-1/osteopontin proteins or polypeptides.

Biologically active portions of a biosynthetic molecules of the present invention are also featured and include molecules sufficiently homologous to or derived from the biosynthetic molecules of the present invention which include less amino acids than the full biosynthetic molecules, and exhibit at least one activity of the biosynthetic molecules. Typically, biologically active portions at least one domain or motif with at least one activity of the biosynthetic molecules. A biologically active portion can be a polypeptide which is, for example, 10, 25, 50, 100 or more amino acids in length.

III. Pharmaceutical Compositions

The nucleic acid molecules, proteins, and biosynthetic molecules (also referred to herein as "active compounds") of the invention can be incorporated into pharmaceutical compositions suitable for administration. Such compositions typically comprise the nucleic acid molecule, protein, or antibody and a pharmaceutically acceptable carrier. As used herein the language "pharmaceutically acceptable carrier" is intended to include any and all solvents, dispersion media, coatings, antibacterial and antifungal agents, isotonic and absorption delaying agents, and the like, compatible with pharmaceutical administration. The use of such media and agents for pharmaceutically active substances is well known in the art. Except insofar as any conventional media or agent is incompatible with the active compound, use thereof in the compositions is contemplated. Supplementary active compounds can also be incorporated into the compositions.

A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. Examples of routes of administration include parenteral, e.g., intravenous, intradermal, subcutaneous, oral (e.g., inhalation), transdermal (topical), transmucosal, and rectal administration. Solutions or suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerine, propylene glycol or other synthetic solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes or multiple dose vials made of glass or plastic.

Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic

water, Cremophor EL™ (BASF, Parsippany, NJ) or phosphate buffered saline (PBS). In all cases, the composition must be sterile and should be fluid to the extent that easy syringability exists. It must be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyetheylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be preferable to include isotonic agents, for example, sugars, polyalcohols such as manitol, sorbitol, sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

Sterile injectable solutions can be prepared by incorporating the active compound (e.g., a Immunomodulin protein or anti-Immunomodulin antibody) in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

Oral compositions generally include an inert diluent or an edible carrier. They can be enclosed in gelatin capsules or compressed into tablets. For the purpose of oral therapeutic administration, the active compound can be incorporated with excipients and used in the form of tablets, troches, or capsules. Oral compositions can also be prepared using a fluid carrier for use as a mouthwash, wherein the compound in the fluid carrier is applied orally and swished and expectorated or swallowed. Pharmaceutically

compatible binding agents, and/or adjuvant materials can be included as part of the composition. The tablets, pills, capsules, troches and the like can contain any of the following ingredients, or compounds of a similar nature: a binder such as microcrystalline cellulose, gum tragacanth or gelatin; an excipient such as starch or lactose, a disintegrating agent such as alginic acid, Primogel, or corn starch; a lubricant such as magnesium stearate or Sterotes; a glidant such as colloidal silicon dioxide; a sweetening agent such as sucrose or saccharin; or a flavoring agent such as peppermint, methyl salicylate, or orange flavoring.

For administration by inhalation, the compounds are delivered in the form of an aerosol spray from pressured container or dispenser which contains a suitable propellant, e.g., a gas such as carbon dioxide, or a nebulizer.

Systemic administration can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active compounds are formulated into ointments, salves, gels, or creams as generally known in the art.

The compounds can also be prepared in the form of suppositories (e.g., with conventional suppository bases such as cocoa butter and other glycerides) or retention enemas for rectal delivery.

In one embodiment, the active compounds are prepared with carriers that will protect the compound against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Methods for preparation of such formulations will be apparent to those skilled in the art. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically

acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Patent No. 4,522,811.

It is especially advantageous to formulate oral or parenteral compositions in dosage unit form for ease of administration and uniformity of dosage. Dosage unit form as used herein refers to physically discrete units suited as unitary dosages for the subject to be treated; each unit containing a predetermined quantity of active compound calculated to produce the desired therapeutic effect in association with the required pharmaceutical carrier. The specification for the dosage unit forms of the invention are dictated by and directly dependent on the unique characteristics of the active compound and the particular therapeutic effect to be achieved, and the limitations inherent in the art of compounding such an active compound for the treatment of individuals.

Toxicity and therapeutic efficacy of such compounds can be determined by standard pharmaceutical procedures in cell cultures or experimental animals, *e.g.*, for determining the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between toxic and therapeutic effects is the therapeutic index and it can be expressed as the ratio LD50/ED50. Compounds which exhibit large therapeutic indices are preferred. While compounds that exhibit toxic side effects may be used, care should be taken to design a delivery system that targets such compounds to the site of affected tissue in order to minimize potential damage to uninfected cells and, thereby, reduce side effects.

The data obtained from the cell culture assays and animal studies can be used in formulating a range of dosage for use in humans. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED50 with little or no toxicity. The dosage may vary within this range depending upon the dosage form employed and the route of administration utilized. For any compound used in the method of the invention, a "therapeutically effective" dose can be estimated initially from cell culture assays. A "therapeutically effective" dose can be further formulated in animal models to achieve a circulating plasma concentration range that includes the IC50 (i.e., the concentration of the test compound which achieves a half-maximal inhibition of symptoms) as determined in cell culture. Such information can be used to more accurately determine useful doses in humans. Levels in plasma may be measured, for example, by high performance liquid chromatography.

The nucleic acid molecules of the invention can be inserted into vectors and used as gene therapy vectors. Gene therapy vectors can be delivered to a subject by, for example, intravenous injection, local administration (see U.S. Patent 5,328,470) or by stereotactic injection (see e.g., Chen et al. (1994) *PNAS* 91:3054-3057). The pharmaceutical preparation of the gene therapy vector can include the gene therapy vector in an acceptable diluent, or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery vector can be produced intact from recombinant cells, e.g., retroviral vectors, the pharmaceutical preparation can include one or more cells which produce the gene delivery system.

The pharmaceutical compositions can be included in a container, pack, or dispenser together with instructions for administration.

IV. Uses and Methods Featuring Biosynthetic Immunomodulatory Molecules of the Present invention

A. Assays

The ability of an stimulatory or inhibitory agent of the invention (*e.g.*, an Eta-1/osteopontin modulator or a biosynthetic immunomodulatory molecule) to modulate an immune response (*e.g.*, to bias an immune response from a type-1 to a type-2 immune response or from a type-2 to a type-1 immune response) can be evaluated using an *in vitro* culture system such as that described herein in the Examples. For example, expression, production or secretion of a cytokine can be determined (*e.g.*, of a type-1 or a type-2 cytokine) or a cytokine profile can be determined (*e.g.*, a type-1 or a type-2 cytokine profile). Immune effector cells (*e.g.*, peripheral blood mononuclear cells) can be cultured in the presence of an stimulatory or inhibitory agent of the invention as described in the examples in a medium suitable for culture of the chosen cells. In the case of assaying for the ability of an inhibitory agent of the invention to modulate an immune response (*e.g.*, an Eta-1/osteopontin inhibitory modulator or an IL-10 component-containing biosynthetic immunomodulatory molecule) it may be necessary to also stimulate cells with a known stimulatory agent. After a period of time (*e.g.*, 24-72 hours), production of cytokine(s) (*e.g.*, at least one type-1 cytokine, at least one type-2 cytokine, a type-1 cytokine profile or a type-2 cytokine profile) is assessed

by determining the level of the cytokine in the culture supernatant as described in the examples. The ability of a stimulatory agent to stimulate cytokine production is evidenced by a higher level of cytokine in the supernatants of cells cultured in the presence of the stimulatory agent compared to the level of cytokine in the supernatant of cells cultured alone or in the presence of a control. The ability of an inhibitory agent to inhibit cytokine production is evidenced by a lower level of cytokine in the supernatants of cells cultured in the presence of both the inhibitory agent and the stimulatory agent compared to the level of cytokine in the supernatant of cells cultured only in the presence of the stimulatory agent.

B. Therapeutic Uses

The present invention provides for both prophylactic and therapeutic methods of treating subjects (*e.g.*, human subjects). In one aspect, the invention provides a method for preventing or treating a disease or a disorder in a subject prophylactically or therapeutically. Administration of a agent prophylactically can occur prior to the manifestation of symptoms of an undesired disease or disorder, such that the disease or disorder is prevented or, alternatively, delayed in its progression. The prophylactic methods of the present invention can be carried out in a similar manner to therapeutic methods described herein, although dosage and treatment regimes may differ.

Another aspect of the invention pertains to methods for treating a subject therapeutically. In one embodiment, the present invention includes methods of modulating an immune response. In particular, modulation of an immune response includes, but is not limited to, modulation of cellular toxicity, modulation of cytokine expression, production or secretion (*e.g.*, enhancement or inhibition of cytokine expression, production or secretion). A preferred embodiment of the invention involves modulation of IL-12, in particular, stimulation of IL-12 using an Eta-1/osteopontin stimulatory modulator or, alternatively, inhibition of IL-12 using an Eta-1/osteopontin inhibitory modulator. Another preferred embodiment of the invention involves modulation of IL-10, in particular, inhibition of IL-10 using an Eta-1/osteopontin stimulatory modulator or, alternatively, stimulation of IL-10 using an Eta-1/osteopontin inhibitory modulator. Accordingly, the present method has therapeutic utility in biasing

an immune response towards, or away from, a type-1 immune response depending upon the desired therapeutic regimen. Such modulatory methods are particularly useful in diseases such as cancer, in immunology, for example, in allergy, organ transplantation and organ rejection. Moreover, the immunomodulatory methods of the present invention can be used to treat an immunocompromized individual to enhance immunity. Uses to increase resistance to viral infection and enhance the rejection of foreign molecules are also within the scope of the present invention. The immunomodulatory methods of the present invention are further useful in wound healing. For example, an enhancement of type-1 immunity in a burn victim, or alternatively, at the burn or wound site, can result in a more rapid immune response, thus preventing infection. The immunomodulatory methods of the present invention are further useful in treating asthma. These various immunomodulatory therapeutic applications are described in further detail in the following subsection.

V. Clinical Applications of the Modulatory Methods of the Invention

The identification of Eta-1/osteopontin as a critical regulator of type 1 immunity allows for selective manipulation of T cell subsets in a variety of clinical situations using the modulatory methods of the invention. The stimulatory methods of the invention (*i.e.*, methods that use an Eta-1/osteopontin stimulatory agent) upregulate the production of the Th1-associated cytokine IL-12 and/or dowregulate the production of the Th2-associated cytokine IL-10, with concomitant promotion of a type 1 immune responses and downregulation of type 2 immune responses. In contrast, the inhibitory methods of the invention (*i.e.*, methods that use an Eta-1/osteopontin inhibitory agent) downregulate the production of the Th1-associated cytokine IL-12 and/or upregulate the production of the Th2-associated cytokine IL-10, with concomitant downregulation of a type 1 immune responses and promotion of type 2 immune responses.

Thus, to treat a disease condition wherein a type 1 immune response is beneficial, a stimulatory method of the invention is selected such that type 1 immune responses are promoted while downregulating type 2 immune responses. Alternatively, to treat a disease condition wherein a type 2 immune response is beneficial, an inhibitory method of the invention is selected such that type 1 immune responses are downregulated while promoting type 2 immune responses. Application of the methods

of the invention to the treatment of disease conditions may result in cure of the condition, a decrease in the type or number of symptoms associated with the condition. either in the long term or short term (*i.e.*, amelioration of the condition) or simply a transient beneficial effect to the subject.

Numerous disease conditions associated with a predominant type 1 or type immune responses have been identified and could benefit from modulation of the type of response mounted in the individual suffering from the disease condition. Application of the immunomodulatory methods of the invention to such diseases is described in further detail below.

A. *Allergies*

Allergies are mediated through IgE antibodies whose production is regulated by the activity of Th2 cells and the cytokines produced thereby. In allergic reactions, IL-4 is produced by Th2 cells, which further stimulates production of IgE antibodies and activation of cells that mediate allergic reactions, *i.e.*, mast cells and basophils. IL-4 also plays an important role in eosinophil mediated inflammatory reactions. Accordingly, the Eta-1/osteopontin stimulatory methods of the invention, which promote type 1 responses and inhibit type 2 responses, can be used to inhibit the type 2 responses (e.g., production of Th2-associated cytokines) in allergic patients as a means to downregulate production of pathogenic IgE antibodies. A stimulatory agent may be directly administered to the subject or cells (*e.g.*, Th0 cells or Th1 cells) may be obtained from the subject, contacted with a stimulatory agent *ex vivo*, and readministered to the subject. Moreover, in certain situations it may be beneficial to coadminister to the subject the allergen together with the stimulatory agent or cells treated with the stimulatory agent to inhibit (*e.g.*, desensitize) the allergen-specific type 2 response. The treatment may be further enhanced by administering other Th1-promoting agents, such as the cytokine IL-12 or antibodies to Th2-associated cytokines (*e.g.*, anti-IL-4 or anti-IL-10 antibodies), to the allergic subject in amounts sufficient to further stimulate a type 1 immune response.

B. *Cancer*

The expression of Th2-promoting cytokines has been reported to be elevated in cancer patients (see *e.g.*, Yamamura, M., *et al.* (1993) *J. Clin. Invest.* 91:1005-1010; Pisa, P., *et al.* (1992) *Proc. Natl. Acad. Sci. USA* 89:7708-7712) and malignant disease is often associated with a shift from Th1 type responses to Th2 type responses along with a worsening of the course of the disease. Accordingly, the stimulatory methods of the invention can be used to promote type 1 responses and inhibit type 2 responses (e.g., the production of Th2-associated cytokines) in cancer patients, as a means to counteract the Th1 to Th2 shift and thereby promote an ongoing Th1 response in the patients to ameliorate the course of the disease. The stimulatory methods can involve either direct administration of a stimulatory agent to a subject with cancer or *ex vivo* treatment of cells obtained from the subject (*e.g.*, Th0 or Th1 cells) with a stimulatory agent followed by readministration of the cells to the subject. The treatment may be further enhanced by administering other Th1-promoting agents, such as the cytokine IL-12 or antibodies to Th2-associated cytokines (*e.g.*, anti-IL-4 or anti-IL-10 antibodies), to the recipient in amounts sufficient to further stimulate a Th1-type response.

C. *Infectious Diseases (e.g., Bacterial or Viral)*

The expression of Th2-promoting cytokines also has been reported to increase during a variety of infectious diseases (including viral and bacterial infectious diseases), including HIV infection, tuberculosis, leishmaniasis, schistosomiasis, filarial nematode infection and intestinal nematode infection (see *e.g.*; Shearer, G.M. and Clerici, M. (1992) *Prog. Chem. Immunol.* 54:21-43; Clerici, M and Shearer, G.M. (1993) *Immunology Today* 14:107-111; Fauci, A.S. (1988) *Science* 239:617-623; Locksley, R. M. and Scott, P. (1992) *Immunoparasitology Today* 1:A58-A61; Pearce, E.J., *et al.* (1991) *J. Exp. Med.* 173:159-166; Grzych, J-M., *et al.* (1991) *J. Immunol.* 141:1322-1327; Kullberg, M.C., *et al.* (1992) *J. Immunol.* 148:3264-3270; Bancroft, A.J., *et al.* (1993) *J. Immunol.* 150:1395-1402; Pearlman, E., *et al.* (1993) *Infect. Immun.* 61:1105-1112; Else, K.J., *et al.* (1994) *J. Exp. Med.* 179:347-351) and such infectious diseases are also associated with a Th1 to Th2 shift in the immune response.

Accordingly, the stimulatory methods of the invention can be used in infectious diseases (caused by bacterial, viral or other pathogenic origins) to promote a type 1 response and inhibit a type 2 response (e.g., the production of Th2-associated cytokines) in subjects with infectious diseases, as a means to counteract the Th1 to Th2 shift and thereby promote an ongoing Th1 response in the patients to ameliorate the course of the infection. The stimulatory method can involve either direct administration of a stimulatory agent to a subject with an infectious disease or *ex vivo* treatment of cells obtained from the subject (*e.g.*, Th0 or Th1 cells) with a stimulatory agent followed by readministration of the cells to the subject. The treatment may be further enhanced by administering other Th1-promoting agents, such as the cytokine IL-12 or antibodies to Th2-associated cytokines (*e.g.*, anti-IL-4 or anti-IL-10 antibodies), to the recipient in amounts sufficient to further stimulate a Th1-type response.

D. *Autoimmune Diseases*

The Eta-1/osteopontin inhibitory methods of the invention can be used therapeutically in the treatment of autoimmune diseases that are associated with a Th2-type dysfunction. Many autoimmune disorders are the result of inappropriate activation of T cells that are reactive against self tissue and that promote the production of cytokines and autoantibodies involved in the pathology of the diseases. Modulation of T helper-type responses can have an effect on the course of the autoimmune disease. For example, in experimental allergic encephalomyelitis (EAE), stimulation of a Th2-type response by administration of IL-4 at the time of the induction of the disease diminishes the intensity of the autoimmune disease (Paul, W.E., *et al.* (1994) *Cell* 76:241-251). Furthermore, recovery of the animals from the disease has been shown to be associated with an increase in a Th2-type response as evidenced by an increase of Th2-specific cytokines (Koury, S. J., *et al.* (1992) *J. Exp. Med.* 176:1355-1364). Moreover, T cells that can suppress EAE secrete Th2-specific cytokines (Chen, C., *et al.* (1994) *Immunity* 1:147-154). Since stimulation of a Th2-type response in EAE has a protective effect against the disease, stimulation of a Th2 response in subjects with multiple sclerosis (for which EAE is a model) is likely to be beneficial therapeutically.

Similarly, stimulation of a Th2-type response in type I diabetes in mice provides a protective effect against the disease. Indeed, treatment of NOD mice with IL-4 (which promotes a Th2 response) prevents or delays onset of type I diabetes that normally develops in these mice (Rapoport, M.J., *et al.* (1993) *J. Exp. Med.* 178:87-99). Thus, stimulation of a Th2 response in a subject suffering from or susceptible to diabetes may ameliorate the effects of the disease or inhibit the onset of the disease.

Yet another autoimmune disease in which stimulation of a Th2-type response may be beneficial is rheumatoid arthritis (RA). Studies have shown that patients with rheumatoid arthritis have predominantly Th1 cells in synovial tissue (Simon, A.K., *et al.*, (1994) *Proc. Natl. Acad. Sci. USA* 91:8562-8566). By stimulating a Th2 response in a subject with RA, the detrimental Th1 response can be concomitantly downmodulated to thereby ameliorate the effects of the disease.

Accordingly, the Eta-1/osteopontin inhibitory methods of the invention, which downregulate type 1 responses (e.g., by inhibition of IL-12 production) can be used to shift the immune response to a type 2 immune response (e.g., stimulating production of Th2-associated cytokines) in subjects suffering from, or susceptible to, an autoimmune disease in which a Th2-type response is beneficial to the course of the disease. The inhibitory method can involve either direct administration of an inhibitory agent to the subject or *ex vivo* treatment of cells obtained from the subject with an inhibitory agent followed by readministration of the cells to the subject. The treatment may be further enhanced by administering other Th2-promoting agents, such as IL-4 or IL-10 itself or antibodies to Th1-associated cytokines (e.g., anti-IL-12 antibodies) to the subject in amounts sufficient to further stimulate a Th2-type response.

In contrast to the autoimmune diseases described above in which a Th2 response is desirable, other autoimmune diseases may be ameliorated by a Th1-type response. Such diseases can be treated using an Eta-1/osteopontin stimulatory agent of the invention (as described above for cancer and infectious diseases). The treatment may be further enhanced by administrating a Th1-promoting cytokine (*e.g.*, IFN-γ) to the subject in amounts sufficient to further stimulate a Th1-type response.

The efficacy of agents for treating autoimmune diseases can be tested in the above described animal models of human diseases (*e.g.*, EAE as a model of multiple sclerosis and the NOD mice as a model for diabetes) or other well characterized animal

models of human autoimmune diseases. Such animal models include the *mrl/lpr/lpr* mouse as a model for lupus erythematosus, murine collagen-induced arthritis as a model for rheumatoid arthritis, and murine experimental myasthenia gravis (see Paul ed., *Fundamental Immunology*, Raven Press, New York, 1989, pp. 840-856). A modulatory (*i.e.*, stimulatory or inhibitory) agent of the invention is administered to test animals and the course of the disease in the test animals is then monitored by the standard methods for the particular model being used. Effectiveness of the modulatory agent is evidenced by amelioration of the disease condition in animals treated with the agent as compared to untreated animals (or animals treated with a control agent).

Non-limiting examples of autoimmune diseases and disorders having an autoimmune component that may be treated according to the invention include diabetes mellitus, arthritis (including rheumatoid arthritis, juvenile rheumatoid arthritis, osteoarthritis, psoriatic arthritis, bacterial arthritis), multiple sclerosis, myasthenia gravis, systemic lupus erythematosis, autoimmune thyroiditis, dermatitis (including atopic dermatitis and eczematous dermatitis), psoriasis, Sjögren's Syndrome, including keratoconjunctivitis sicca secondary to Sjögren's Syndrome, alopecia areata, allergic responses due to arthropod bite reactions, Crohn's disease, aphthous ulcer, iritis, conjunctivitis, keratoconjunctivitis, ulcerative colitis, asthma, allergic asthma, cutaneous lupus erythematosus, scleroderma, vaginitis, proctitis, drug eruptions, leprosy reversal reactions, erythema nodosum leprosum, autoimmune uveitis, allergic encephalomyelitis, acute necrotizing hemorrhagic encephalopathy, idiopathic bilateral progressive sensorineural hearing loss, aplastic anemia, pure red cell anemia, idiopathic thrombocytopenia, polychondritis, Wegener's granulomatosis, chronic active hepatitis, Stevens-Johnson syndrome, idiopathic sprue, lichen planus, Crohn's disease, Graves ophthalmopathy, sarcoidosis, primary biliary cirrhosis, uveitis posterior, and interstitial lung fibrosis.

E. *Transplantation Rejection*

While graft rejection or graft acceptance may not be attributable exclusively to the action of a particular T cell subset (*i.e.*, Th1 or Th2 cells) in the graft recipient (for a discussion see Dallman, M.J. (1995) *Curr. Opin. Immunol.* 7:632-638), numerous studies have implicated a predominant Th2 response in prolonged graft

survival or a predominant Th1 response in graft rejection. For example, graft acceptance has been associated with production of a Th2 cytokine pattern and/or graft rejection has been associated with production of a Th1 cytokine pattern (see *e.g.*, Takeuchi, T. *et al.* (1992) *Transplantation* 53:1281-1291; Tzakis, A.G. *et al.* (1994) *J. Pediatr. Surg.* 29:754-756; Thai, N.L. *et al.* (1995) *Transplantation* 59:274-281). Additionally, adoptive transfer of cells having a Th2 cytokine phenotype prolongs skin graft survival (Maeda, H. *et al.* (1994) *Int. Immunol.* 6:855-862) and reduces graft-versus-host disease (Fowler, D.H. *et al.* (1994) *Blood* 84:3540-3549; Fowler, D.H. *et al.* (1994) *Prog. Clin. Biol. Res.* 389:533-540). Still further, administration of IL-4, which promotes Th2 differentiation, prolongs cardiac allograft survival (Levy, A.E. and Alexander, J.W. (1995) *Transplantation* 60:405-406), whereas administration of IL-12 in combination with anti-IL-10 antibodies, which promotes Th1 differentiation, enhances skin allograft rejection (Gorczynski, R.M. *et al.* (1995) *Transplantation* 60:1337-1341).

Accordingly, the Eta-1/osteopontin inhibitory methods of the invention, which inhibit type 1 immune responses, can be used to shift the bias toward type 2 immune responses in transplant recipients to prolong survival of the graft. The inhibitory methods can be used both in solid organ transplantation and in bone marrow transplantation (*e.g.*, to inhibit graft-versus-host disease). The inhibitory method can involve either direct administration of an inhibitory agent to the transplant recipient or *ex vivo* treatment of cells obtained from the subject with an inhibitory agent followed by readministration of the cells to the subject. The treatment may be further enhanced by administering other Th2-promoting agents, such asIL-4 or IL-10 itself or antibodies to Th1-associated cytokines (e.g., anti-IL-12 antibodies), to the recipient in amounts sufficient to further stimulate a Th2-type response.

F. *Other Disorders for Upregulation of Type 1 Immune Responses*

In addition to the foregoing, there are numerous other disorders in which it can be beneficial to upregulate (i.e., bias toward) type 1 immune responses using the Eta-1/osteopontin stimulatory methods of the invention, as follows:

Burn associated sepsis is associated with the excess production of the type 2 cytokine IL-10. Accordingly, use of an Eta-1/osteopontin stimulatory method of

the invention to promote type 1 responses (e.g., by upregulating IL-12 production and/or downregulating IL-10 production) can be beneficial in the treatment of burn-associated sepsis.

Immunodeficiency disorders often are associated with a lack of, or insufficient, type 1 immunity. Accordingly, immunodeficiency disorders such as AIDS, bone marrow transplant-associated immunodeficiency, and chemotherapy-associated immunodeficiencies, can be treated using an Eta-1/osteopontin stimulatory method of the invention to promote type 1 responses (e.g., by upregulating IL-12 production and/or downregulating IL-10 production).

Any of the foregoing treatments may be further enhanced by administering other Th1-promoting agents, such as the cytokine IL-12 or antibodies to Th2-associated cytokines (*e.g.*, anti-IL-4 or anti-IL-10 antibodies), to the recipient in amounts sufficient to further stimulate a Th1-type response.

G. *Other Disorders for Downregulation of Type 1 Immune Responses*

In addition to the foregoing, there are numerous other disorders in which it can be beneficial to downregulate (i.e., bias away from) type 1 immune responses (and bias toward type 2 immune responses) using the Eta-1/osteopontin inhibitory methods of the invention, as follows:

Granulomatous disorders result from excessive type 1 responses (discussed further in Example 1) and experiments have demonstrated that in the absence of Eta-1 (e.g., in an Eta-1 deficient animal) sarcoid-type granulomas fail to form. Accordingly, use of an Eta-1/osteopontin inhibitory method of the invention to downregulate type 1 responses (e.g., by downregulating IL-12 production and/or upregulating IL-10 production) can be beneficial in the treatment of granulomatous disorders.

Herpes Simplex Virus Keratitis (HSK) results from corneal infection by Herpes Simplex Virus-1 (HSV-1) that leads to a destructive autoimmune inflammatory reaction that depends on the production of IL-12 and that is inhibited by IL-10 (discussed further in Example 2). Accordingly, use of an Eta-1/osteopontin inhibitory method of the invention to downregulate type 1 responses (e.g., by downregulating IL-

12 production and/or upregulating IL-10 production) can be beneficial in the treatment of HSK.

Bacterial arthritis is associated with excessive type 1 responses subsequent to bacterial infection. Accordingly, use of an Eta-1/osteopontin inhibitory method of the invention to downregulate type 1 responses (e.g., by downregulating IL-12 production and/or upregulating IL-10 production) can be beneficial in the treatment of bacterial arthritis.

Any of the foregoing treatments may be further enhanced by administering other Th2-promoting agents, such asIL-4 or IL-10 itself or antibodies to Th1-associated cytokines (e.g., anti-IL-12 antibodies), to the recipient in amounts sufficient to further promote a Th2-type response.

In addition to the foregoing disease situations, the modulatory methods of the invention also are useful for other purposes. For example, the stimulatory methods of the invention (*i.e.*, methods using a stimulatory agent) can be used to stimulate production of Th1-promoting cytokines (*e.g.*, IL-12) *in vitro* for commercial production of these cytokines (*e.g.*, cells can be contacted with the stimulatory agent *in vitro* to stimulate IL-12 production and the IL-12 can be recovered from the culture supernatant, further purified if necessary, and packaged for commercial use).

Furthermore, the modulatory methods of the invention can be applied to vaccinations to promote either a Th1 or a Th2 response to an antigen of interest in a subject. That is, the agents of the invention can serve as adjuvants to direct an immune response to a vaccine either to a Th1 response or a Th2 response. For example, to stimulate an antibody response to an antigen of interest (*i.e.*, for vaccination purposes), the antigen and an Eta-1 inhibitory agent of the invention can be coadministered to a subject to bias the response towards type 2 responses (e.g., antibody production) to the antigen in the subject, since Th2 responses provide efficient B cell help and promote IgG1 production. Alternatively, to promote a cellular immune response to an antigen of interest, the antigen and a stimulatory agent of the invention can be coadministered to a subject to promote a Th1 response to the antigen in a subject, since Th1 responses favor the development of cell-mediated immune responses (*e.g.*, delayed hypersensitivity responses). The antigen of interest and the modulatory agent can be formulated together

into a single pharmaceutical composition or in separate compositions. In a preferred embodiment, the antigen of interest and the modulatory agent are administered simultaneously to the subject. Alternatively, in certain situations it may be desirable to administer the antigen first and then the modulatory agent or vice versa (for example, in the case of an antigen that naturally evokes a Th2 response, it may be beneficial to first administer the antigen alone to stimulate a Th2 response and then administer an Eta-1/osteopontin stimulatory agent, alone or together with a boost of antigen, to shift the immune response to a Th1 response).

VI. Tumor Immunity, Irradiated Tumor Cells

The present invention also features methods of modulating tumor immunity. Such methods are based, at least in part, on the understanding that tumor cells are capable of escaping destruction by a subject's immune system, *i.e.*, are capable of escaping the subject's natural immune responses. Accordingly, in one embodiment, the invention features a method of modulating tumor immunity which involves contacting a tumor cell with an Eta-1/osteopontin modulator such that tumor immunity is modulated. A preferred embodiment features a method of enhancing a type 1 response to a tumor cell which involves contacting the cell with an Eta-1 stimulatory agent such that a type 1 response against the cell is enhanced. Another preferred embodiment features a method of enhancing a type 1 response to a tumor cell which involves contacting the cell with an Eta-1 stimulatory agent such that a type 1 response against the cell is stimulated (*e.g.*, is stimulated by the tumor cell so contacted). In one embodiment, the tumor cell is contacted *in vivo*. In another embodiment, the tumor cell is contacted *ex vivo*. For example, tumor cells can be isolated from the subject and cultures in the presence of an Eta-1/osteopontin modulator (*e.g.*, an Eta-1 stimulatory agent). In another embodiment, the method can further include administering (*e.g.*, readministering) the cells to the patient. In yet another embodiment, the cells are transfected in culture with an isolated nucleic acid Eta-1/osteopontin modulator (*e.g.*, a nucleic acid molecule encoding Eta-1/osteopontin or a biologically active fragment thereof, or encoding a biosynthetic molecule of the present invention. Methods for transfecting cells, vectors and the like are as described herein (for example, in section I.B.)

In yet another embodiment, the invention features methods of modulating tumor immunity (*e.g.*, methods of enhancing a type 1 response against a tumor or tumor cells) which further includes the step of irradiating the tumor cells (*e.g.*, before or after contacting with an Eta-1 modulator) such that the cells are incapable of replicating once administered to the patient. In yet another embodiment, the method further features the step of transfecting the cells with GMCSF. Also featured are tumor cells treated with an Eta-1/osteopontin modulator of the present invention. In one embodiemnt, the invention features tumor cells transfected with an Eta-1-encoding nucleic acid molecule or nucleic acid molecule encoding a biologically active fragment of Eta-1/osteopontin. In another embodiment, the invention features tumor cells transfected with Eta-1/osteopontin and GMCSF. In yet another embodiemnt, the invention features a tumor cells transfected with a nucleic acid molecule which encodes a biosynthetic immunomodulatory molecule of the preset invention.

The present invention is further illustrated by the following Examples which in no way should be construed as further limiting. The entire contents of all of the references (including literature references, issued patents, and published patent applications) cited throughout this application are hereby expressly incorporated by reference.

Exemplification

Examples 1-4 demonstrate an essential role for Eta-1/osteopontin in regulating immune responses (*e.g.*, type-1 immune responses) *in vivo*. These examples further demonstrate the applicability of administering Eta-1/osteopontin as an *in vivo* approach to regulating such immune responses.

Example 1: Eta-1/opn-dependent modulation of type-1 immunity (*e.g.*, in a classical granulomatous response) *in vivo* in control, nude, cytokine-deficient and Eta-1/opn-deficient mice

An early and essential step in type-1 immunity is the migration of macrophages/dendritic cells to the site of infection, and subsequent activation of the recruited macrophages, a process that is controlled by CD4 T-cells. Eta-1/osteopontin is the most abundantly expressed mRNA transcript after activation of CD4 cells (Patarca *et al.* (1989) *J. Exp. Med.* 170:145-161; Weber *et al.* (1997) *Proc. Assoc. Am. Physicians* 109:1-9; Rittling and Denhardt (1999) *Exp. Nephrol.* 7:103). Production of IL-12 by activated macrophages/dendritic cells and reception of the IL-12 signal by CD4 cells are subsequent critical steps in this process. Although an interaction between CD40 ligand on activated T-cells and CD40 on macrophages can induce IL-12 expression (Scheicher *et al.* (1995) *Eur. J. Immunol.* 25, 1566; Macatonia *et al.* (1995) *J. Immunol.* 154:5071; Murphy (1998) *Curr. Opin. Immunol.* 10:226) this interaction also induces the inhibitory IL-10 cytokine and may not suffice for induction of IL-12 *in vitro* (Ria *et al.* (1998) *Eur. J. Immunol.* 28:2003) or for sustained levels of IL-12 that follow viral infection *in vivo* (Sharma *et al.* (1998) *J. Immunol.* 161:5357).

The Eta-1 gene is expressed in T cells early in the course of bacterial infections (within 48 hours), and interaction of its protein product with macrophages can induce inflammatory responses (Singh *et al.* (1990) *Exp. Med.* 171:1931; Yu *et al.* (1998) *Proc. Assoc. Am. Physicians* 110:50; Denhardt and Noda (1998) *J. Cell. Biochem. Suppl.* 30/31:92). Genetic resistance to infection by certain strains of Rickettsia may depend on Eta-1-dependent attraction of monocytes into infectious sites and acquisition of bacteriocidal activity (Patarca *et al.* (1993) *Crit. Rev. Immunol.* 13:225; Jerrells and Osterman (1981) *Infect. Immun.* 31:1014); the granulomatous responses characteristic of sarcoidosis and tuberculosis are associated with high levels of Eta-1 expression (Nau *et al.*(1997) *Proc. Natl. Acad. Sci. U.S.A.* 94:6414; O'Regan *et al.* (1999) *J. Immunol.* 162:1024).

Granuloma formation in these human diseases is a cellular consequence of type-1 immunity (Patarca *et al.* (1993) *Crit. Rev. Immunol.* 13:225; Jerrells and Osterman (1981) *Infect. Immun.* 31:1014). Accordingly, a valuable *in vivo* animal model for studying type-1 immune responses involves inducing sarcoid-type granulomas in mice

by injection of polyvinyl pyrrolidone (PVP) (van den Bogert *et al.* (1986) *Virchows Arch.* 51:39). Because certain murine models of parasite-induced granulomas may reflect a mixture of type-2 and type-1 immunity (O'Garra (1998) *Immunity* 8:275), the importance of IL-12-dependent type-1 immunity in this murine model of granuloma formation was first established.

The granulomatous response was first measured in control (C57BL/6 (+/+)) and nude (C57BL/6 *nu/nu*) mice. PVP-dependent granulomas were formed by injecting mice subcutaneously above the right hind limb with 500 µl of 0.5% PVP. After 5 days, mice were killed, and tissue was extracted for histologic analysis. Figure 1A depicts the data as (1) the mean number of granulomas per high-power field (HPF) (X200 magnification); (2) as the mean number of cells per granuloma after examination of 50 to 80 HPF per mouse; and (3) as the product of these two indices, termed "granuloma burden". (Error bars indicate 1 SEM.). An intense granulomatous response was provoked shortly after subcutaneous injection of PVP into C57BL/6 (+/+) but not C57BL/6 *nu/nu* strains of mice (see *e.g.*, top two bars of Figure 1A).

It was next determined whether Eta-1 administration could reconstitute the granulomatous response in C57BL/6 *nu/nu* strains of mice. For experiments using purified Eta-1/opn to modulate immunity, Eta-1 is prepared as follows. To generate naturally-produced (native) Eta-1/opn, MC3T3E1 cells or Ar5v T-cells were grown in defined media (consisting of DME/H12 supplemented with pyruvate, insulin, transferrin, selenium and ethanolmine) in 5% CO_2 at 37°C. Media was dialized against PBS and concentrated using a Millipore tangential flow system applied to Millipore LC100 equipped with a DEAE-Memsep 1000 cartridge and developed in a discontinuous gradient of 0 to 1 M NaCl in phosphate buffer, pH 7.4. Eta-1/opn-containing fractions were pooled (the major Eta-1/opn peak eluted at 0.26 M salt), concentrated by ultrafiltration, chromatofocused on mono P columns (Pharmacia) at pH 8.2, developed with polybuffer 74 (Pharmacia) and the major Eta-1/opn fraction eluted from monobeads at pH 4.6. The protein was judged pure by several criteria including SDS electrophoresis and amino acid sequence analysis (both N-terminal and internal peptide analysis). Mass spectroscopic analysis revealed a peak centered around a mass of 35,400 that was highly phosphorylated (11 mols of phosphate/mol of protein), O-glycosylation but not N-glycosylation, and no measurable sulfate. For

dephosphorylated, naturally-produced Eta-1/opn. 5 mg of purified Eta-1/opn was incubated with 6 units (60 units/mg) type II potato acid phosphatase in 20 mM phosphate buffer pH 4.8 at 37°C for 2 h. After adjusting the pH to 8.2, dephosphorylated protein was applied to a chromatofocusing column and the major peak eluted at a pH of 5.1: amino acid analysis of the protein revealed a phosphate content of less than 1 mol/mol protein.

When C57BL/6 *nu/nu* mice were coinjected with PVP inoculum plus 10 μg of Eta-1, the granulomatous response was partially restored, demonstrating that the Eta-1/opn gene product can partially substitute for activated T lymphocytes in this setting. The granulomatous response was likewise determined in cytokine-deficient (C57BL/6 IL-$12^{-/-}$ and C57BL/6 IL-$10^{-/-}$) mice. The granulomatous response was diminished by 70 to 80% in C57BL/6 IL-$12^{-/-}$ mice and was enhanced two- to three-fold in C57BL/6 IL-$10^{-/-}$ mice.

It was then asked whether mice deficient in Eta-1 secondary to targeted gene mutation formed granulomas after PVP injection. C57BL/6 × 129/SV Eta-$1^{-/-}$ mice generated as described by Rittling *et al.* (1998) *J. Bone Miner. Res.* 13:1101, were compared to either Eta-$1^{+/+}$ littermates or age-matched C57BL/6 × 129/SV mice as controls. Histological analysis was performed on tissue sections from PVP injection sites. Briefly, samples were fixed in 10% buffered formalin and embedded in paraffin. Embedded sampled were sectioned into 4- to 5-μm serial sections and stained with hematoxylin and eosin. Images were captured with a Sony DXC-970MD video camera and Optima 5.2 Histomorphometric analysis software.

Histological analysis of tissue sections at PVP injection sites at 20X, 100X and 400X magnifications showed granulomatous infiltrations of mononuclear cells in subcutaneous dermal and subdermal areas in Eta-$1^{+/+}$ mice 5 days after injection of PVP, PBS, or PVP + 5 μg purified Eta-1. By contrast, Eta-$1^{-/-}$ mice did not develop a detectable granulomatous response after challenge with PVP. However, coinjection of purified Eta-1 with PVP the partially restored the granulomatous response in Eta-$1^{-/-}$ mice (these experiments and Figure 1A).

Analysis of surface antigens expressed by cells within granulomas in the various mouse strains was done with monoclonal antibodies to Mac-1, B220, CD3, and BP-55 (a neutrophil marker). Histologic analysis of granulomas formed in Eta-$1^{-/-}$ mice

and in Eta-1$^{-/-}$ mice reconstituted with purified Eta-1 revealed a similar macrophage-dominant cellular infiltrate. About 85% of granulomatous cells in both cases were Mac-1^{+}, whereas 5 to 10% were CD3^{+} T cells or B220^{+} B cells. BP-55^{+} neutrophils, which were only a minor component (1 to 2%) of granulomas in these mice, increased 5- to 10-fold in granulomas firmed in IL-10$^{-/-}$ mice. Eta-1$^{-/-}$ mice also displayed defective granulomatous responses to injection of collagen and latex, consistent with reports that human T cells resident in sterile granulomas have high expression of Eta-1 (Nau *et al.* (1997) *Proc. Natl. Acad. Sci. U.S.A.* 94:6414; O'Regan *et al.* (1999) *J. Immunol.* 162:1024).

Lastly, the cytokine expression profiles were determined for cells from lymph nodes draining the site of granulomas in Eta-1$^{+/+}$ and Eta-1$^{-/-}$ mice. Briefly, PVP-dependent granulomas were formed as described above. After 5 days, mice were killed, and local lymph nodes were obtained for cytokine expression. Cytokine expression was measured 48 hours after incubation with PVP (2×10^6 cells per well). Restimulation of lymph nodes draining subcutaneous sites of PVP injection in Eta-1$^{-/-}$ mice and control mice with PVP revealed impaired IL-12 and interferon-γ (IFN-γ) responses. The IL-12 response was reduced by ~95%, and the IFN-γ response of Eta-1$^{-/-}$ mice was reduced by 90% in comparison to Eta-1$^{+/+}$ controls (Figure 1C).

Example 2: Eta-1/opn-dependent modulation of type-1 immunity and destructive type-1 autoimmune responses *in vivo* in herpes simplex virus-type 1 (HSV-1) infected control and Eta-1/opn-deficient mice

A second valuable *in vivo* animal model for studying type-1 immune responses involves inoculating mice (*e.g.*, corneal inoculation) with herpes simplex virus-1 ("HSV-1"). Inoculation with HSV-1 leads to delayed type immune responses in mice that can manifest as classical footpad swelling (Foster *et al* (1986) *Clin. Immunol. Immunopathol.* 40:313-325). Corneal HSV-1 infection can also lead to a destructive autoimmune inflammatory reaction, Herpes Simplex Keratitis (HSK), initiated by CD4 cells that recognize a viral peptide mimic of a murine corneal self-antigen (Zhao *et al.* (1998) *Science* 279:1344; Avery *et al.* (1995) *Nature* 376:431). This inflammatory response depends on the production of IL-12 and is inhibited by IL-10 (Streilein *et al.* (1997) *Immunol. Today* 18:443; Daheshia *et al.* (1997) *J. Immunol.* 159:1945).

Eta-1$^{-/-}$ mice were infected in the right eye with $4x10^6$ plaque-forming units (PFU) HSV-1 (KOS strain) and challenged five days later in the left footpad with $1x10^5$ PFU of UV-inactivated HSV-1 (KOS). Eta-1$^{-/-}$(opn$^{-/-}$) mice infected by HSV-1 ($4x10^6$ PFU *via* the cornea) fail to develop a significant DTH response after footpad challenge with 10^5 pfu HSV-1, in contrast to the strong DTH response of Eta-1$^{+/+}$(opn$^{+/+}$) controls (Figure 2A).

The numbers of T cells and proportions of T cell subsets in the thymus and peripheral lymphoid tissues of Eta-1$^{-/-}$ mice were similar to Eta-1$^{+/+}$ littermates. T and B cell subsets in Eta-1$^{-/-}$ and Eta-1$^{+/+}$ littermates were as follows: C57BL/6 × 129 Eta-1$^{+/+}$ spleen, 93.7×10^6 total cells (30.8% CD3, 19.8% CD4, 11% CD8, and 49.7% B220); C57BL/6 × 129 Eta-1$^{-/-}$ spleen, 82.6×10^6 cells (27.8% CD3, 18.8% CD4, 9.0% CD8, and 55.5% B220); C57BL/6 × 129 Eta-1$^{+/+}$ lymph node, 32.0×10^6 cells (82.4% CD3, 42.8% CD4, 34.2% CD8, and 12.8% B220); and C57BL/6 × 129 Eta-1$^{-/-}$ lymph node, 21.9×10^6 cells (82.8% CD3, 49.3% CD4, 28.4% CD8, and 11.2% B220). Moreover, T cells from Eta-1$^{-/-}$ and Eta-1$^{+/+}$ mice expressed levels of CD44 and CD62 that were not distinguishable. Although the T cell numbers and proportions were similar in Eta-1$^{-/-}$ and Eta-1$^{+/+}$ mice, the possibility existed that defective antiviral DTH response in Eta-1$^{-/-}$ mice might reflect a subtle alteration in lymphocyte or macrophage development. Accordingly, the effects of acute *in vivo* depletion of Eta-1 with a neutralizing antibody were tested in the Eta-1$^{-/-}$ mice. The neutralizing antisera LF-123 (Fisher *et al.* (1995) *Acta Orthop. Scand.* 66:61) or control normal rabbit serum were injected at 25 μg per dose per day, starting 2 days before injection. On day 0, mice were infected with HSV-1 (KOS) and rechallenged 5 days later. The right and left footpads of each mouse were measured 24 hours after rechallenge, and specific swelling (left versus right footpad) is shown in Figure 2B. Administration of antibody to Eta-1 (LF-123) immediately before and repeatedly after HSV-1 infection efficiently inhibited the DTH response upon rechallenge.

In a second experiment, Eta-1$^{-/-}$ and control mice (Eta-1$^{+/-}$) were subjected to ocular challenge with virus. As shown in Figure 2C, Eta-1$^{-/-}$ mice failed to develop significant HSK within 2 weeks after corneal inoculation with HSV-1 in contrast to the severe HSK developed within this period by control littermates (Eta-1$^{+/-}$) (*i.e.*, 65% of control Eta-1$^{+/-}$ mice developed HSK, Figure 2C). Similar results were

obtained when the experiment was repeated using BALB/cBγJ mice and CB-17 mice in addition to Eta-1$^{-/-}$ and Eta-1$^{+/+}$ mice, as demonstrated in Figure 2D. Furthermore, skewing of the cell numbers in Eta-1/opn knockout mice after challenge with HSV-1 was diminished compared to control mice in which the increase of CD8$^+$ cells is consistent with a Th-1 response.

Moreover, T-cells from Eta-1$^{-/-}$ mice are not impaired in their proliferative response to irradiated virus plus antigen presenting cells. The right superficial cervical draining lymph nodes of Eta-1$^{-/-}$ mice and Eta-1$^{+/+}$ littermate controls were harvested 15 days after infection of the right eye with 4×10^6 PFU of HSV-1 (KOS). Cells from these lymph nodes (2×10^6 cells per well) were incubated in the presence of 4×10^7 PFU of ultraviolet (UV)-inactivated HSV-1 (KOS). The proliferative response of lymph node cells from HSV-1-infected Eta-1$^{+/+}$ and Eta-1$^{-/-}$ mice measured by ^{3}H-thymidine incorporation at 72 h was 20.9×10^3 and 18.7×10^3 cpm, respectively. Furthermore, the absence of DTH does not reflect a general impairment of the immune system in these mice since clonal expansion followed by apoptosis after superantigen (SEB) injection were indistinguishable from wild-type mice. T cell expansion followed by apoptosis after superantigen (50 µg of staphylococcal enterotoxin B) intraperitoneal injection into Eta-1$^{-/-}$ and Eta-1$^{+/+}$ mice was indistinguishable at 3 days: +/+ V$_\beta$8$^+$ CD4 cells (percentage of total spleen) increased from 3.6 to 5%; -/- V$_\beta$8$^+$ CD4 cells increased from 3.2 to 5.5%; +/+ V$_\beta$6$^+$ CD4 cells increased from 2.3 to 2.6%; -/- V$_\beta$6$^+$ CD4 cells increased from 2.5 to 2.6%.

Although cells from the draining lymph nodes of virus-infected Eta-1$^{-/-}$ and Eta-1$^{+/+}$ mice respond equally well to HSV-1 according to [^{3}H]-thymidine incorporation after viral restimulation *in vitro*, they differed conspicuously according to their cytokine profiles. Briefly, cells were isolated and restimulated with HSV-1 (KOS) as described above. Supernatants were harvested 48h later and IL-10 and IL-12 p40 cytokine levels were measured by sandwich ELISA using OptIEA antibody sets (Pharmingen, La Jolla CA). IL-4 was measured after stimulation of draining lymph node cells by plate-bound anti-CD3. Cells from Eta-1$^{-/-}$ mice produced high levels of IL-10 and IL-4 but markedly reduced levels of IL-12, compared with Eta-1$^{+/+}$ controls (Figure 2E) and splenic macrophages from virus-infected Eta-1$^{+/+}$ but not Eta-1$^{-/-}$ mice continued to produce IL-12 ten days after infection. In contrast with the sterile granulomatous response, IFN-γ levels

were not reduced in Eta-1$^{-/-}$ mice after HSV-1 viral infection, consistent with an IL-12-independent pathway to IFN-γ production that may depend on virally induced IFN-α/β production (Oxenius *et al.* (1999) *J. Immunol.* 162-965; Cousens *et al.* (1999) *J. Exp. Med.* 189:1315). Moreover, expression of IL-2 by lymph node and spleen T lymphocytes from Eta-1$^{-/-}$ and Eta-1$^{-/+}$ littermates in response to immobilized antibody to CD3 was indistinguishable between the C57BL/6 × 129/SV Eta-1$^{-/-}$ and C57BL/6 × 129/SV Eta-1$^{-/-}$ mice. These cytokine profiles suggest that Eta-1/osteopontin expression normally may imprint the *in vivo* ratio of IL-12 and IL-10 cytokines that dictates a type-1 immunity.

Example 3: Treatment of HSV-1 infected mice with anti-Eta-1/osteopontin antibodies significantly downregulates type-1 immunity and destructive type-1 autoimmune responses

In a similar experiment to those described above, the levels of HSV-1-specific DTH reactions were measured 24 hours after footpad challenge in Cal20 mice that had been primed five days earlier by corneal inoculation of 4 x 10^4 to 4 x 10^7 plaque forming units ("pfu") of UV-inactivated HSV-1 and treated with anti-osteopontin antibody, LF-123 (Fisher *et al.* (1995) *Acta Orthop. Scand.* 66:61), or control serum every 48 hours. Mice treated only with control serum exhibited classical footpad swelling when footpads were measured 24 hours after challenge. By contrast, mice treated with LF-123 serum exhibited significantly diminished footpad swelling, indicating that neutralization of osteopontin significantly inhibited footpad swelling.

Table I: Specific footpad swelling in Cal20 mice in the presence of rabbit serum or anti-osteopontin antibody following HSV-1 inoculation (increasing pfus).

HSV-KOS	rabbit serum	LF-123
$4x10^4$	0.04 mm	0.07
$2x10^5$	0.1	0.05
$4x10^5$	0.42	0.03
$4x10^6$	0.57	0.13
$4x10^7$ (24 hr)	1.114	0.6175

Moreover, as described above, HSV-1 infection of murine cornea leading to HSK results in corneal inflammation and destruction within 14 days after viral inoculation. Cal20 mice that were treated with anti-osteopontin antibody every 48 hours after challenge had reduced severity and incidence of HSK compared to mice injected with rabbit serum.

Table II: HSK in Cal20 mice in the presence of rabbit serum or anti-osteopontin antibody following HSV-1 inoculation

	Incidence*	Severity*
Cal20	77.77% 100%	2.11 3.1
Cal20 rabbit serum	50% 50%	2 2.25
Cal 20 LF-123	60% 60%	1.6 2

*day 11/day 14

Example 4: Eta-1/osteopontin-dependent modulation of protective immune responses following infection (*e.g., listeria monocytogenes* infection) *in vivo* in control and Eta-1/osteopontin-deficient mice

The murine response to *Listeria monocytogenes* is an experimental cornerstone of our understanding of the early events leading to type-1 immunity after microbial infection (Unanue (1997) *Immunol. Rev.* 158:11) and depends on early macrophage production of IL-12 and downstream expression of IFN-γ (Tripp *et al.* (1993) *Proc. Natl. Acad. Sci. U.S.A.* 90:3725; C. S. Tripp, Gately *et al.* (1994) *J. Immunol.* 152:1883; Tripp *et al.* (1995) *J. Immunol.* 155:3427). Accordingly, the ability of Eta-$1^{-/-}$ mice to mount a protective immune response after *Listeria* infection was investigated.

Listeria infection and cytokine production were as follows. Virulent *L. monocytogenes* (strain 1778, American Type Culture Collection (ATCC) designation 43251) was grown in trypticase soy broth, and 10^3 colony-forming units (CFU), a sublethal dose for this strain of *L. monocytogenes*, were injected intravenously into C57BL/6 (B6), B6-IL-$12^{-/-}$, B6-IL-$10^{-/-}$, B6 x 129-Eta-$1^{-/-}$, and B6 x 129-Eta-$1^{-/+}$ mice (Unanue (1997) *Immunol. Rev.* 158:11; Stordeur *et al.* (1995) *Mol. Immunol.* 32:233

(1995); Stordeur and Goldman (1998) *Int. Rev. Immunol.* 16:501). The titer of viable bacteria in the inoculum and in organ homogenates (*e.g.*, liver and spleen) was determined by plating 10-fold serial dilutions on trypticase soy agar plates. Plates were incubated at 37°C. and the numbers of CFU were counted after 24 hours. Eta-$1^{-/-}$ mice contained liver-associated *Listeria*-infected cysts that were apparent 4 (first experiment) and 5 (second experiment) days after infection (also seen in IL-$12^{-/-}$ mice). At 5 days (second experiment) *Listeria* infection was also evident in spleen homogenate of Eta-$1^{-/-}$ mice. These data demonstrate that Eta-$1^{-/-}$ mice were defective in their ability to clear *L. monocytogenes* after systemic infection, similar to the defect in IL-$12^{-/-}$ mice.

Restimulation of spleen cells from Eta-$1^{-/-}$ and Eta-$1^{+/+}$ mice with heat-killed bacteria revealed that cells from the former mice had reduced IFN-γ responses. Briefly, Spleen cells (4×10^6/ml) from four to five C57BL/6 × 129 Eta-$1^{+/+}$ or four to five C57BL/6 × 129 Eta-$1^{-/-}$ mice that had been intravenously inoculated 5 days earlier with 10^3 CFU were stimulated with heat-killed *L. monocytogenes* (2×10^8 CFU/ml) 96 hours before IFN-γ measurement by an OptEIA™ ELISA kit (PharMingen). 25.5 ± 6.5 ng/ml of IFN-γ were produced by spleen cells from Eta-$1^{+/+}$ mice in comparison with 3.2 ± 1.2 ng/ml of IFN-γ from Eta-$1^{-/-}$ mice.

The data presented in Examples 1-4 clearly demonstrate a role for Eta-1/osteomodulin in a variety of type-1 immune responses and demonstrate that type-1 immunity can be modulated by administration of purified Eta-1. The data presented in Examples 1-4 further indicate that Eta-1/osteopontin expression potentially effects type-1 immunity through regulation of the IL-12 and IL-10 cytokine ratio.

Example 5: Eta-1/osteopontin-dependent modulation of type-1 immunity cytokine profiles *in vitro*

This example further defines the role of Eta-1/opn in modulating immune effector cells, in particular, by demonstrating the ability of Eta-1/opn to modulate type-1 cytokine secretion *in vitro* (*e.g.*, in isolate peritoneal macrophages). Resident peritoneal macrophages were isolated from normal mice and treated with increasing amounts of purified Eta-1/opn. Briefly, peritoneal macrophages were obtained by peritoneal lavage (2x10 ml PBS) of C57BL/6 mice. Contaminating red cells were eliminated by hypotonic

lysis with ACK buffer. Cells were plated at 10^5/100 µl in 96-well plates and non-adherent cells were washed off after 2 hours. Adhered cells were then incubated for 48 hours with increasing concentrations of purified Eta-1/opn in serum-free medium and levels of IL-10 and IL-12 p75 in the supernatant were determined by ELISA (Figure 3A). Briefly, supernatant was withdrawn at the indicated time points for analysis of IL-10 or IL-12 p70 using commercial ELISA kits (R & D Systems). At the end of the incubation, the cells were tested for viability by propidium iodide incorporation (>98%) and their purity was confirmed by staining with fluorescence-conjugated anti-Mac1 antibody (>98%).

Treatment of cells with Eta-1/opn resulted in the secretion of as much as 400 pg/ml of IL-12 at 48 hours whereas IL-10 production was not detected (Figure 3A). Eta-1/opn-dependent induction of IL-12 secretion from macrophages was not due to contamination with endotoxin as Limulus ameboid lysate assay indicated that purified Eta-1/opn contained less than 1ng/g endotoxin. Moreover, quantities of endotoxin that escape detection in the limulus ameboid lysate assay do not contribute to biologic activity of Eta-1/opn because the IL-12 response of macrophages derived from C3H.HeJ mice (which are defective in endotoxin receptor-mediated signaling) was not impaired compared to other strains.

Next, resident peritoneal macrophages ($5x10^5$/ml) were treated with either 5 pmol/ml Eta-1/opn, 30 ng/ml LPS or 500 U/ml IL-4 and IL-12/IL-10 detected by ELISA at increasing times post-induction (Figure 3B). While LPS stimulation of these resident peritoneal macrophages induced both IL-12 (about 250 pg/ml) and IL-10 (about 100 pg/ml) and while IL-4 predominantly caused production of IL-10, Eta-1/opn selectively lead to secretion of IL-12. The failure of Eta-1/opn to induce IL-10 was somewhat surprising since other cytokines that activate macrophages (*e.g.*, TNFα, IL-1,IL-2,IL-3 and IL-6 all stimulate IL-10 secretion (Stordeur *et al.* (1995 *Mol. Immonol.* 32:233; Stordeur and Goldman (1998) *Int. Rev. Immunol.* 16:501), and lippopolysaccharide (LPS) stimulation of these resident peritoneal macrophages induced both IL-12 (~250 pg/ml) and IL-10 (~100 pg/ml).

Further analysis showed that Eta-1/opn actively suppressed IL-10 secretion by resident peritoneal macrophages stimulated with IL-4 (Figure 3C). Briefly, macrophages were activated with IL-4 (500 U/ml x 1 hour) before addition of Eta-1/opn (5

pmol/ml) for an additional 48 h before measurement of IL-12 and IL-10 by ELISA. In some groups, anti-IL-12 (R & D Systems, Minneapolis, MN) was added at a final concentration of 2 μg/ml. As shown in Figure 3C, IL-4-dependent induction of macrophage IL-10 was inhibited by the addition of Eta-1/opn, but this effect was not altered by anti-IL-12 neutralizing antibody, suggesting a direct mode of action.

Moreover, Eta-1 actively suppressed the LPS dependent IL-10 response of resident peritoneal macrophages (Figure 3D). Briefly, macrophages were activated with LPS (30 ng/ml) for 1 hour before addition of Eta-1 (5 nM) for an additional 48 hours and consecutive measurement of IL-12 and IL-10 by ELISA. Assays were performed in quadruplets, and each point represents the mean and standard error (error bars) of two independent experiments.

Examples 6-12 define the functional domains of Eta-1/osteopontin and map various Eta-1/osteopontin-dependent activities to their respective domains. These Examples also define various bioactive fragments of Eta-1 for modulating immune effector cell activation (*e.g.*, cell motility, spreading, cytokine and metalloproteinase secretion). These examples also describe the phosphorylation dependence of various Eta-1/osteopontin-dependent activities.

Additional Materials and Methods for Examples 6-12

Cell lines: A31 is an integrin $\alpha_v\beta_3$, CD44 murine embryonic fibroblast clone derived from Balb 3T3 cells (CCL-163, ATCC). A31 cells transfected with CD44 (A31.C1) or A31 mock-transfectants were generated as described (Weber *et al.* (1996) *Science* 26:271:509-512). MH-S is a macrophage cell line that was derived by SV40 transformation from an adherent cell enriched population of alveolar macrophages (CRL-2019, ATCC). MT-2/1 is a thymus-derived macrophage from a Balb/c mouse that was immortalized by infection with retroviral vector. It expresses CD44 and integrin $\alpha_v\beta_3$.

Eta-1/opn purification and cleavage: To generate recombinant Eta-1/opn, GST-Eta-1/opn fusion protein was expressed in *E. coli*, digested with factor Xa, and purified by affinity chromatography as described (3 refs). For phosphorylated recombinant Eta-1/opn, GST-Eta-1/opn (5 mg) was incubated with10 μg of Golgi kinase

for 2 h before passage through a GSH-Sepharose column and elution from GSH-Beads with 100 U of factor Xa. The eluate was applied to a chromatofocusing column and eluted from the resin with polybuffer 74 as described above. The major peak eluted at pH 4.6 and phospho-amino acid analysis of the recovered protein revealed a phosphoserine content of 16 mol of phosphate/mol protein and 0.8 mols of phosphothreonine/mol protein. Native Eta-1/opn were prepared as described above. Thrombin cleaves Eta-1/opn into two fragments following the arginine in the sequence VVYGLR in Eta-1/opn (*e.g.*, amino acid residues 162-168 of SEQ ID NO:2), an N-terminal fragment ("Eta-1/opn NT) containing the RGD motif and a C-terminal fragment ("Eta-1/opn CT"). Thrombin cleavage and phosphorylation of either the dephosphorylated native protein or recombinant Eta-1/opn was accomplished by human thrombin (Sigma Chemicals), Golgi kinases or purified casein kinase II or casein kinase I.

Chemotaxis: Directed migration of cells was determined in multi-well chemotaxis chambers as described (Weber *et al.* (1996) *Science* 26:271:509-512). Briefly, two-well culture plates (Transwell) with polycarbonate filters (pore size 8-12 μm) separating top and bottom wells were coated with 5μg fibronectin. 2 X 10^5 cells were added to the upper chamber and incubated at 37° C in the presence or absence of Eta-1/opn in the lower chamber. After 4 h, the filters were removed, fixed in methanol, stained with hematoxylin and eosin and cells that had migrated to various areas of the lower surface were counted microscopically. Controls for chemokinesis included 200 ng of the appropriate form of osteopontin in the top well. All assays were done in triplicates and are reported as mean ± standard deviation.

Haptotaxis: Haptotaxis of monocytic cell lines to Eta-1/opn or fragments of Eta-1/opn was assayed using a Boyden chamber. The lower surface or both sides of polycarbonate filters with 8 μm pore size were coated with the indicated amounts of Eta-1/opn. 2 X 10^5 cells were added to the upper chamber, and incubated at 37° C in the absence of any factors in the lower chamber. After 4 h, the filters were removed, fixed in methanol and stained with hematoxylin and eosin. Cells that had migrated to the lower surface were counted under a microscope. All assays were done in triplicates and are reported as mean ± standard deviation.

Cell attachment and spreading: 24-well plates were coated over night at 4°C with 10 µg/ml of the indicated ligand then blocked for 1 h at room temperature with 1-10 mg/ml BSA in PBS. To preserve the integrity of adhesion receptors, MH-S monocytic cells were harvested from subconfluent cultures by non-enzymatic cell dissociation solution (Sigma, St Louis MO). Cells were washed twice with PBS and resuspended at a concentration of 1 X 10^5 cell/ml of sterile Ca^{2+} and Mg^{2+} free PBS supplemented with 0.1% BSA and 1 mM sodium pyruvate. 5 x 10^3 to 5 x 10^4 cells were incubated in each well and, after 1 h at 37°C, the wells were washed 3 times with 0.5 ml PBS to remove non-adherent cells, fixed in 10% buffered formalin, 1% paraformaldehyde, or 1% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) at room temperature for ~1 hour then stained with toluidene blue and hematoxylin. The total number of attached or spread cells in each well were counted microscopically using a Nikon Eclipse microscope equipped with a Sony digital Camera. Total number of attached or spread cells were quantitated using Optima 5.2 image analysis system. Each experiment was done in triplicates and is reported as mean ± SEM. To minimize variability inherent to cell attachment studies, cells were scored as attached only when a defined nucleus was observed, accompanied by a transition from round to cuboidal cell morphology. Round cells that are loosely attached with no defined nucleus were scored as non-attached. These cells can be removed with repeated washes. The viability of the cells was measured before and after the termination of the experiments and only data from experiments with greater than 95 % cell viability were used. Further, under the conditions used in these experiments, cell attachment was temperature dependent, inhibitable by trypsin treatment and not affected by inhibitors of protein synthesis or secretion. Cell spreading was determined by membrane contour analysis and was scored according to increase in cell volume/surface area. In some experiments, cell spreading was also assessed by the formation of stress fibers. Each experiment was performed in quadruplicate wells and repeated 3 times.

Example 6: Effects of Various Eta-1/opn Domains on Cellular Chemotaxis

This example describes the domain-specific effects of Eta-1/osteopontin on chemotaxis of immune effector cells.(*e.g.*, monocytes).

Stable CD44 transfectants of $\alpha_v\beta_3$ fibroblasts (Weber *et al.* (1996) *Science* 26:271:509-512) were used to examine the interaction of CD44 with osteopontin. Chemotactic activity of Eta-1/opn or Eta-1/opn fragments was tested in a modified Boyden chamber (Weber *et al.* (1996) *Science* 26:271:509-512). Purified natural osteopontin exerted chemotactic activity for the MH-S monocyte cell line. Moreover, the C-terminal thrombin cleavage product but not the N-terminal cleavage fragment mediated chemotaxis of A31.C1 (CD44 stably transfected cells), but not mock-transfected A31.MLV cells, confirming the dependence of cell migration on the expression of CD44. The C-terminal fragment of Eta-1/opn also induced chemotaxis of macrophage cell line MH-S as efficiently as intact Eta-1/opn, whereas the N-terminal 30 kDa Eta-1/opn fragment was inactive.

Table III: Chemotactic Response of MH-S Cells to Eta-1/opn

	LOWER CHAMBER			
UPPER CHAMBER	PBS	Eta-1/opn	Eta-1/opn CT	Eta-1/opn NT
PBS	56±10	312±56*	478±98*	71±21
Eta-1/opn	34±7	168±24	305±50	36±19
Eta-1/opn CT	9±4	88±24	220±38	26±5
Eta-1/opn NT	63±11	287±60	409±55	14±5

* $P<0.01$

Moreover, equimolar mixtures of both fragments displayed activity similar to that of the 28 kDa C-terminal fragment alone. These results, taken together, indicate that the chemotactic domain of Eta-1/opn resides in the 28 kDa C-terminal part of the molecule. This C-terminal fragment-mediated activity could further be inhibited by Eta-1/opn fragments and various modulators of receptor interaction.

Table IV: Inhibition of Monocyte Chemotaxis

	MI Eta-1/opn	MI Eta-1/opn CT
Control	13.3 ± 1.9	9.6 ± 1.9
+ GRGDS† (1mM)	10.6 ± 1.3	7.7 ± 1.6
+ anti CD44 (0.1 μg)	7.8 ± 0.7**	4.6 ± 0.7*
+ anti β3 (0.1 μg)	9.8 ± 0.6	12.1 ± 2.1
+ Eta-1/opn NT	12.3 ± 2.0	9.6 ± 1.9
+ Eta-1/opn CT	6.3 ± 0.7*	3.7 ± 1.6*
+Eta-1/opn	4.4 ± 0.6*	2.6 ± 0.2*

* P<0.01 ** P<0.05 † (SEQ ID NO:11)

Previous investigations of stable transfectants of A31 cells showed that the interaction of Eta-1/opn with CD44 depended on expression of CD44 splice variants 3-6 (Weber *et al.* (1996) *Science* 26:271:509-512), which characterize activated lymphocytes (Arch *et al.* (1992) *Science* 257:682-685). More recent studies indicate that A31 cells transfected with the standard form of CD44 (lacking variant exons) do not bind Eta-1/opn.

Example 9: Effects of Various Eta-1/opn Domains of Hapotaxis

This example describes the domain-specific effects of Eta-1/osteopontin on haptotaxis of immune effector cells (*e.g.*, monocytes).

Cells can move up a gradient of immobilized ligand, a process referred to as haptotaxis. This cell crawling may occur on vessel walls or in the interstitium. Therefore, the contribution to cell motility of interactions between immobilized Eta-1/opn, Eta-1/opn fragments, and integrin receptors was assessed as follows. The ability of the immobilized ligand to induce monocyte haptotaxis was judged by cell migration through poly-carbonate filters. Eta-1/opn induced monocyte migration that was mainly directional (*i.e.*, the cells responded to a positive gradient of bound Eta-1/opn), and thus haptotactic and was inhibited by GRGDS (SEQ ID NO:11) and antibody to the β_3 chain

of integrins but not by antibody to CD44. Data are expressed as migratory index (cells migrating in response to Eta-1/opn/cells migration in response to buffer). Values are expressed as mean ± SEM.

Table V: Hapotactic Response of Monocytes to Eta-1/opn

	Eta-1/opn Bound to Upper Side			
Eta-1/opn Bound to Lower Side	0 pmol	30 pmol	90 pmol	150 pmol
0 μg	1.0 ± 0.15	1.5 ± 0.2	1.8 ± 0.1	0.6 ± 0.2
1	5.2 ± 0.35	1.5 ± 0.3		
3	7.8 ± 0.4		2.1 ± 0.3	
5	9.3 ± 0.8			2 ± 0.1

Table VI: Inhibition of Monocyte Hapotaxis

	MI Eta-1/opn	MI Eta-1/opn-NT
control	9.8 ± 0.9	6.1 ± 0.7
+ GRGDS† (1mM)	3.6 ± 1*	2.1 ± 0.2*
+ anti CD44 (0.1 μg)	7.8 ± 0.7	5.5 ± 0.3
+ anti integrin β3	4.8 ± 0.6*	1.6 ± 0.1*

* P<0.01 † (SEQ ID NO:11)

Table VII: Effects of Eta-1/opn Phosphorylation on Hapotaxis and Chemotaxis

	Phosphorylated Eta-1/opn		Unphosphorylated Eta-1/opn	
	Hapotactic Index	Chemotactic Index	Hapotactic Index	Chemotactic Index
Control	1 ± 0.1	1.3 ± 0.3	1 ± 0.3	1 ± 0.2
Eta-1/opn	9.8 ± 0.9*	13.3 ± 1.9*	3.6 ± 0.6**	11.4 ± 1.8*
Eta-1/opn NT	1.8 ± 0.7	0.9 ± 0.1	0.6 ± 0.2	0.9 ± 0.2
Eta-1/opn CT	1.6 ± 0.5	9.6 ± 1.9*	1.3 ± 0.6	9.6 ± 1.9*
NT10k	4.2 ± 1.1**	1.1 ± 0.2	1.8 ± 0.9	1.1 ± 0.1
rEta-1/opn	-	-	1.5 ± 0.1	10.5 ± 2.2*
rEta-1/opn (GK)	12.6 ± 2.1*	10.4 ± 1.6*	-	-
rEta-1/opn (CKII)	8 ± 1.8*	10.3 ± 1.6*	-	-
rEta-1/opn (CKI)	10 ± 1.9*	9.9 ± 2.3*	-	-
rEta-1/opn (PKG)	0.8 ± 0.4	8.7 ± 2.0*	-	-

Example 8: Effects of Eta-1/opn and Various Eta-1/opn Domains on Cellular Spreading

This example describes the domain-specific and phosphorylation-dependent effects of Eta-1/osteopontin on the spreading of immune effector cells (*e.g.*, the spreading of monocytes).

Macrophage spreading on extracellular matrix proteins depends, in part, on engagement of their integrin receptors. MH-S cells attached and spread on immobilized phosphorylated Eta-1/opn whereas MH-S cells plated on unphosphorylated Eta-1/opn did not spread (as determined microscopically). Spreading of the MH-S macrophage cell line on immobilized native Eta-1/opn is mediated by the RGD-containing N-terminal thrombin cleavage fragment but not by the C-terminal fragment and is reversed by addition of soluble GRGDS (SEQ ID NO:11) but not control GRGES (SEQ ID NO:12) peptide (Figure 4A).

Moreover, phosphorylation of recombinant Eta-1/opn is required for this activity. rEta-1/opn was phosphorylated with the indicated kinases as previously described (Ashkar, 1993, 1993b, 1995, Salih,1997). rEta-1/opn (GK), recombinant Eta-1/opn phosphorylated with golgi kinases isolated for mouse calvarial cells (14 mol of

phosphate/mol protein); rEta-1/opn (CKII) rEta-1/opn phosphorylated with casein kinase II (9 mol phosphate/mol protein) rEta-1/opn (CKI) phosphorylated with casein kinase I (11 mol phosphate/mol protein) rEta-1/opn (PKG) recombinant Eta-1/opn phosphorylated with cGMP dependent protein kinase (3 mol phosphate/mol protein). Since none of the sites are phosphorylated 100% the mol phophate/mol protein does not reflect the total number of sites phosphorylated.

Table VIII: Spreading Indices Indicate the Effect of Phosphorylation of Recombinant Eta-1/opn (rEta-1/opn) and Dephosphorylation of Native Eta-1/opn

	Phosphorylated Eta-1/opn	Unphosphorylated Eta-1/opn
Control	1 ± 0.3	1 ± 0.1
Eta-1/opn	10 ± 2.1*	2 ± 0.7
Eta-1/opn NT	11 ± 1.6*	11 ± 1.5
Eta-1/opn CT	2 ± 0.7	2 ± 0.8
NT10k	5 ± 2.4**	7 ± 0.5
REta-1/opn	-	2.4 ± 0.3
rEta-1/opn (GK)	11 ± 1.7*	-
rOEta-1/opn (CKII)	12 ± 3.1*	-
rEta-1/opn (CKII)	8 ± 2.6*	-
Eta-1/opn(PKG)	1 ± 0.1	-

Cleavage of Eta-1/opn with thrombin exposes the RGD motif and may enhance its cell attachment properties (Senger *et al.* (1995)). Mutagenesis of the RGD sequence substantially reduced attachment of melanoma cells (Smith *et al.* (1998)), tumor cells, and gingival fibroblasts (Xuan *et al.* (1995)) demonstrating the necessity of this motif.

While the RGD sequence is necessary for integrin binding, it is not specific for a particular integrin receptor. Eta-1/opn may be secreted in nonphosphorylated (Kubota *et al.* (1989) *Biochem. Biophys. Res. Comm.* 162:1453-1459: Chambers *et al.* (1992) *Anticancer Res.* 12:43-47: Barak-Shalom *et al.* (1995) *Comp. Biochem. Physiol.* 111:49-59: and Chang and Prince (1993) *Cancer Res.* 53:2217-2220) and phosphorylated forms that contain up to 28 phosphate residues

(Sorensen and Peterson (1994) *Biochem. Biophys. Res Comm.* 198:200-205; Salih *et al.* (1995) *Ann. NY Acad. Sci.* 760:357-360; Salih *et al.* (1997) *J. Biol. Chem.* 272:13966-13973. Phosphorylation is functionally important because it may determine whether Eta-1/opn associates with the cell surface or with the extracellular matrix and it may be essential for integrin-mediated cell adhesion. Therefore, phosphorylation of the molecule may provide selectivity of integrin binding. Phosphorylation has to occur at specific sites because Golgi kinases and casein kinases I or II can activate Eta-1/opn whereas protein kinases A or G phosphorylate the recombinant molecule but do not confer integrin binding.

In a second experiment, MH-S cells attached to, but did not spread on phosphorylated and unphosphorylated PNGRGDSLAYGLR (SEQ ID NO:13) synthetic peptides. In an attempts to define an N-terminal peptide capable of support attachment and spreading, partial tryptic, chemotryptic and Asp-N endopeptidase digestion of Eta-1/opn was performed. None of these, however, resulted in the isolation of an active peptide. A 10-kD fragment isolated from a Lys-C digest was found to be active. NK10 has the NH_2-terminal sequence QETLPSN (SEQ ID NO:14) and is predicted to terminate at the thrombin cleavage site. This 10-kD fragment also contains ~5 mol of phosphate per 1 mol of peptide at seven potential phosphorylation sites. NK10 was capable of mediating the spreading of macrophages at approximately 40 % (mol/mol) the activity of the larger N-terminal thrombin fragment (Figure 4B). Upon dephosphorylation of this peptide spreading activity is lost, but can be regained by rephosphorylation with Golgi kinases. Earlier studies which showed that RGD-containing peptides can confer function may have induced non-specific effects through multiple integrin receptors. These data demonstrate that the RGD motif is necessary but not sufficient to mediate specific Eta-1/opn activity, phosphorylation in defined sites is also needed.

Example 9: Eta-1/osteopontin-dependent modulation of type-1 immunity cytokines *via* distinct receptors on immune effector cells (*e.g.*, macrophages)

As shown in Examples 6-8, Eta-1/opn interaction with macrophages is mediated through two distinct functional receptors. Engagement of CD44 mediates

chemotactic migration and interaction with $\alpha_v\beta_3$ integrin causes haptotaxis, adhesion and spreading.

To determine whether distinct macrophage receptors were responsible for the type-1 cytokine production by Eta-1/opn stimulated macrophages, fragments from a Lys-C digest of Eta-1/opn were analyzed for the ability to stimulate IL-12 secretion. The 10 kDa NK10 proteolytic fragment from the N-terminal portion of Eta-1/opn containing the integrin binding site was found to be sufficient to induce macrophage IL-12 expression (Figure 5A).

In contrast to IL-12 induction, inhibition of IL-10 depends on engagement of the CD44 receptor: Figure 5B shows that Eta-1/opn-dependent inhibition of IL-4-induced production of IL-10 was reversed by anti-CD44 (KM81, purified from ATCC hybridoma TIB 241, described in Mayake *et al.* (1990) *J. Exp. Med.* 171:477-488) but not anti-integrin β_3 antibody (Pharmingen, described in Schultz and Armant (1995) *J. Biol. Chem.* 270:11522). Moreover, macrophages from $CD44^{-/-}$ mice are resistant to Eta-1/opn inhibition of the IL-10 response. Figure 5C shows that secretion of IL-12 in response to Eta-1/opn was not impaired in macrophages from mice that are deficient in the CD44 gene and cells from C3H.HeJ mice (which do not respond to endotoxin) displayed the same levels of induction as control mice. Conversely, while the inhibition of IL-10 secretion was not affected in C3H.HeJ mice or in C57Bl/6 mice, it was abrogated in $CD44^{-/-}$ mice.

Example 10: Phosphorylation of Eta-1/osteopontin is necessary for engagement of integrin receptors on macrophages leading to IL-12 production but not for ligation of CD44 leading to IL-10 inhibition

Eta-1/opn is secreted in nonphosphorylated and phosphorylated forms (ref.). Phosphorylation may allow Eta-1/opn to associate with the cell surface rather than the extracellular matrix (refs) through a contribution to integrin binding. In contrast, serine phosphorylation of recombinant Eta-1/opn is not required for CD44-dependent interactions leading to chemotactic migration (ref.). To determine whether phosphorylation of Eta-1/opn might affect its ability to regulate cytokine expression, phosphorylated and dephosphorylated Eta-1/opn were tested for their ability to affect IL-12/IL-10 secretion.

Figure 6A demonstrates that dephosphorylated purified naturally-produced Eta-1/opn abolished IL-12 stimulatory activity whereas phosphorylation of recombinant Eta-1/opn at specific sites restores activity. Secretion of IL-12 was measured by ELISA after culture of resident peritoneal macrophages with 6 pmol/ml of dephosphorylated natural Eta-1/opn (dpEta-1/osteopontin), recombinant Eta-1/opn (rEta-1/osteopontin) or recombinant phosphorylated Eta-1/opn (rEta-1/osteopontin~P) for 6 h in defined medium at 37°C. Dephosphorylated native Eta-1/opn and recombinant (unphosphorylated) Eta-1/opn does not induce IL-12 production but retains inhibitory activity for IL-10. Recombinant Eta-1/opn phosphorylation with Golgi kinases (rEta-1/osteopontin~P) conferred IL-12 inducing activity while similar levels of phosphorylation by PKA and PKC did not restore this activity.

Although recombinant Eta-1/opn lacking phosphate groups cannot induce IL-12, this molecule retains inhibitory activity for the macrophage IL-10 response (Figure 6B). Dephosphorylation of native Eta-1/opn resulted in loss of IL-12 inducing activity, while phosphorylation of (inactive) recombinant Eta-1/opn restored this function. There is abundant evidence that phosphorylation can regulate the biological activity of intracellular enzymes and their substrates; these results indicate that serine phosphorylation can also provide molecular information that regulates the biological activity of a secreted protein.

Figure 7 depicts cytokine profiles for macrophages after engagement by phosphorylated versus unphosphorylated Eta-1/opn, as well as by Eta-1/opn fragments. TNFα, TGFβ, and the type-1 cytokine IL-12 as well as the type-1 cytokine IL-10 were determined by commercial ELISA kits. (For induction of TGFβ cells were cultured on the indicated ligand for 6 h in defined media at 37° C in a humidified atmosphere.) Cytokine secretion data is presented as fold induction over resting values. Ligation of integrin receptors (*e.g.*, by native Eta-1/opn, recombinant phosphorylated Eta-1/opn, N-terminal fragment or NK10) on macrophages caused predominantly secretion of IL-12, TNFα, TGFβ but not IL-10 or IL-1α (*e.g.*, a type-1 cytokine profile).

Example 12: Signal Transduction Pathways Associated with Eta-1/opn-Mediated Functions Including Chemotaxis, Hapotaxis and Cell Spreading

This example demonstrates that distinct cellular signaling mechanisms are activated by association of the two key functional domains of Eta-1/osteopontin with their respective receptors on macropages.

Three biological response phenotypes have been observed in association with ligation of CD44 and integrin $\alpha_v\beta_3$: chemotaxis, cell crawling, and activation (after spreading on substrate). Accordingly, it was tested whether these functions were distinguishable on the level of intracellular signal transduction. Signal transduction mechanisms were initially examined through the use of specific chemical inhibitors at the following final concentrations: 50 mM for cycloheximide, PKA inhibitors H89 at lmM and, H7 at 20 uM, Inhibitors of PI pathway Wortmannin at 10 nM, tyrosine kinase inhibitors genistein at 25 uM for, PKC inhibitor chelerythrine at 20 uM, Casein Kinase II inhibitor quercetin at 6 mM. In all experiments using these compounds cells were preincubated for 0.5 hours with the inhibitors before start of the experiment. Cell viability was determined by trypan blue exclusion on cell samples before and after the termination of the experiments. Cell viability in all reported experiments was > 95 %. Microfilament disruption was carried out by preincubation of the cell cultures for one hour in 50 uM cytochalasin D. Microtubule dissociation was carried out by pre-incubation of the cultures for 6 hours in 1 μM colchicine. All compounds were suspended in either DMSO or absolute ethanol and were added to the culture media at 1:1000 dilution. Controls were carried out with the corresponding vehicle. In separate experiments in which PKC and PKA were chemically activated, 50 ng/ml phorbol 12-myristate 13-acetate and 10^5 M of forskolin were used respectively. In these experiments treatments were for 2 hours.

In order to delineate the mechanisms mediating differential activities of Eta-1/opn following ligation of its two groups of receptors, the effects of protein kinase inhibitors, cytoskeletal disrupting agents, and toxins were tested on the macrophage responses. Eta-1/opn mediated chemotaxis is diminished by the G-protein inhibitor pertussis toxin but not by inhibitors of protein kinase C or A, in contrast haptotaxis is not affected by pertussis toxin or protein kinase A inhibitors but is inhibited by the protein kinase C inhibitor chelerythrine.

Table IX: Inhibition of Monocyte Hapotaxis

	MI Eta-1/opn	MI Eta-1/opn
Control	9.8 ± 0.9	6.1 ± 0.7
+ Wortmanin (10nM)	10.1 ± 2.2	8.8 ± 1.3
+ Chelerythrine (20μM)	3.3 ± 0.2*	1.3 ± 0.1*
+ Genistein (25μM)	3.2 ± 0.5**	2.0 ± 0.3
+ PT	10.6 ± 1.1*	9.4 ± 1.5*
+ H 7 (20μM)	10.8 ± 1.7	6.9 ± 2.0
+ Cytocholasin D (1μM)	1.1 ± 0.2*	2.7 ± 0.1*

* P<0.01

Table X: Inhibition of Monocyte Chemotaxis

	MI Eta-1/opn	MI Eta-1/opn CT
control	13.3 ± 1.9	9.6 ± 1.9
+ Wortmanin (10nM)	12.6 ± 2.5	10.5 ± 1.6
+ Chelerythrine (20μM)	3.1 ± 0.5*	1.9 ± 0.8*
+ Genistein (25μM)	6.6 ± 1.1**	4.3 ± 0.7
+ PT	2.3 ± 0.21*	1.6 ± 0.3*
+ H 7 (20μM)	14.1 ± 2.7	8.7 ± 1.2
+ Cytocholasin D (1μM)	1.8 ± 0.9*	2.2 ± 0.3*

*P<0.01 **P<0.05

Ligation of integrin receptors by Eta-1/opn may lead to dephosphorylation of Src in chicken osteoblasts and recombinant Eta-1/opn may phosphorylate paxillin, tensin, and p125 focal adhesion kinase in ras-transformed NIH3T3 cells. G proteins are linked to ligation of CD44 by Eta-1/osteopontin. Ligation of integrin $\alpha_v\beta_3$ initially leads to activation of PKC (see *e.g.*, Figures 8 and 9). After cell spreading, the cytoskeleton rearranges and a second integrin-associated signal transduction component, phosphatidylinositol 3-kinase, is activated (Figure 4B). Spreading of macrophages on Eta-1/opn is inhibited by chelerythrine and by inhibitors of the phosphatidylinositol pathway consistent with earlier reports that engagement of integrin $\alpha_v\beta_3$ on osteoclasts by Eta-1/opn leads to activation of phosphatidylinositol 3-

hydroxyl kinase . Direct measurement of phosphorylation of phosphatidylinositol 3-kinase is in accord with the inhibitor-based observations. The selective inhibition of macrophage responses argues against a toxic effect of these inhibitors.

The data in Example 12 demonstrate that CD44-dependent chemotaxis is associated with a signal transduction pathway that involves G-protein, while integrin-dependent haptotaxis is mediated by a pathway involving protein kinase C. Once a cell has spread, phosphatidylinositol signaling is integrated as a second component into integrin-dependent signaling. Distinct macrophage phenotypes induced by Eta-1/opn can be separated on the level of signal transduction using G-protein, protein kinase C, and phosphatidylinositol 3-kinase as biochemical markers.

Example 11: Domain-Specificity and Phosphorylation-Dependence of Induction of Metalloprotease Secretion by Eta-1/osteopontin

Because cell spreading is often associated with cellular activation, an investigation was made into whether the interaction between phosphorylated Eta-1/opn and macrophages leads to additional signs of macrophage activation including secretion of metalloproteinases and cytokines.

For metalloprotease secretion assays, MH-S cells were stimulated for 6 hours with either phosphorylated or unphosphorylated Eta-1/opn at a concentration of 10 µg/ml in serum-free defined medium. In order to visualize the secreted metalloproteases, gelatin zymograms were performed. Briefly, cell culture supernatant was collected after 6 hours of culture, concentrated 5 times and resuspended in 200 µl zymogram buffer (40 mM Tris, pH 7.5) before addition to Laemmli sample buffer and electrophoresis in 10% polyacrylamide gels impregnated with 1mg/ml gelatin. Following electrophoresis, gels were incubated for 30 min at 37°C in 50 ml of 50 mM Tris-HCl buffer, pH 8.0, containing 2% Triton-X 100 and 10 mM $CaCl_2$ to remove the SDS, followed by incubation for 18 h in 50 mM Tris-HCl buffer, pH 8.0, containing 5 mM $CaCl_2$. After staining the gels with Coomassie Brilliant Blue, gelatin and casein degrading enzymes were identified as clear bands against a dark blue background.

MMP-9 and MMP-2 were both visible in the samples stimulated with natural Eta-1/opn or with phosphorylated rEta-1/opn. Control MH-S cells were incubated with serum-free defined medium. MMP9 but not MMP2 was stimulated by the N-terminal

fragment of osteopontin, while the C-terminal fragment of Eta-1/opn had little or no stimulatory activity. MMP-9 induction could be inhibited by GRGDS (SEQ ID NO:11) but not GRGES (SEQ ID NO:12). Dephosphorylation of Eta-1/opn with acid phosphatase abolished the stimulatory activity of Eta-1/opn. Similarly, rEta-1/opn had no stimulatory activity. The results demonstrate that only phosphorylated Eta-1/opn had a stimulatory effect the gelatinolytic activity secreted by MH-S cells.

Definition of the functional domains of Eta-1/opn in the examples described above represents an important step in understanding this process and is critical for the rational development of Eta-1/opn analogs that antagonize or mimic discrete biological activities of the parent molecule. Examples 13-14 describe the generation and testing of such analogs.

Example 13: Generation of Biosynthetic Immunomodulatory Molecules That Stimulate IL-12

A first generation osteopontin-derived biosynthetic molecule was engineered based on the isolation of a domain of osteopontin sufficient to impart IL-12 stimulatory activity when isolated from the naturally-occurring polypeptide. Figure 10 depicts the amino acid and encoding nucleic acid sequence of such a molecule, termed immunomodulin-1, based on its ability to modulate immune responses. In particular, the biosynthetic immunomodulin-1 molecule depicted in Figure 10 has the ability to bias an immune response from a type-2 response to a type-1 response.

Example 14: Generation of Biosynthetic Immunomodulatory Molecules That Inhibit IL-10

A first generation osteopontin-derived biosynthetic molecule was engineered based on the isolation of a domain of osteopontin sufficient to impart IL-10 inhibitory activity when isolated from the naturally-occurring polypeptide. Figure 11 schematically depicts the structure of such a molecule, termed immunomodulin-2, based on its ability to modulate immune responses. In particular, the biosynthetic immunomodulin-2 molecule depicted in Figure 11 has the ability to bias an immune response from a type-2 response to a type-1 response.

Example 15: Testing of Immunomodulin-1 and Immunomodulin-2 *in vitro* and *in vivo*

Immunomodulin-1 and Immunomodulin-2 were tested for their ability to stimulate and/or inhibit cytokine secretion, in particular for their ability to stimulate and/or inhibit secretion of IL-12 and/or IL-10. As shown in Figure 12, Immunomodulin-1 is capable of stimulating IL-12 secretion by macrophages to levels greater than those induced by LPS. Moreover, as demonstrated in Figure 13, IL-10 is capable of inhbiting IL-4 induced IL-10 secretion by macrophages. The data demonstrate that Immunomodulin-1 and –2 are capable of biasing an immune response towards a type-1 response *in vitro*.

In order to test the ability of Immunomodulin-2 to bias an immune response *in vivo* from a type-2 to a type-1 response, C57blk mice were sensitized with a single intraperitoneal injection of 0.1 μg/ml poke weed adsorbed to 2 mg aluminum hydroxide. Animals were challenged at 7 or 14 days following sensitization with either aerosol poke weed (1μg/ml in an atomizer) or *via* subcutaneous injection of 0.05 μg/ml poke weed in phosphate-buffered saline (PBS). Plasma levels of IgE were determined by ELISA using antibodies to mouse IgE. Immunomodulin-2 was injected intraperitoneally (100 μl at a concentration of 10 μg/ml in PBS). Plasma concentrations of IgE were determined 3 and 14 days after injection (Figure 14).

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

What is claimed:

1. A method of modulating a type-1 immune response in a subject comprising administering to said subject an Eta-1/osteopontin modulator such that the type-1 immune response is modulated.

2. The method of claim 1, wherein the Eta-1/osteopontin modulator stimulates Eta-1/osteopontin activity and the type-1 immune response is potentiated.

3. The method of claim 1, wherein the Eta-1/osteopontin modulator inhibits Eta-1/osteopontin activity and the type-1 immune response is downregulated.

4. The method of claim 1, wherein the subject is a human subject.

5. The method of claim 1, wherein the Eta-1/osteopontin modulator is administered in a therapeutically effective amount.

6. The method of claim 1, further comprising monitoring the type-1 response in said subject.

7. The method of claim 6, wherein monitoring the type-1 response comprises determining the level of a detectable indicator of the type-1 response.

8. The method of claim 7, wherein monitoring the type-1 response further comprises comparing the level of the detectable indicator to a control.

9. A method of potentiating a type-1 immune response in a patient comprising:

(a) selecting a patient suffering from a disorder that would benefit from a potentiated type-1 immune response: and

(b) administering to said patient an Eta-1/osteopontin stimulatory modulator such that the type-1 immune response is potentiated.

10. The method of claim 9, wherein the disorder is selected from the group consisting of burn-associated sepsis, bacterial infection, viral infection, parasitic infection, mycoplasma infection, fungal infection, cancer, immunodeficiency disorders, AIDS, bone marrow transplant-related immunodeficiency, chemotherapy-related immunodeficiency and allergy.

11. A method of downregulating a type-1 immune response in a patient comprising:

(a) selecting a patient suffering from a disorder that would benefit from a downregulated type-1 immune response; and

(b) administering to said patient an Eta-1/osteopontin inhibitory modulator such that the type-1 immune response is downregulated.

12. The method of claim 11, wherein the disorder is selected from the group consisting of bacterial arthritis, granulomatous disorder, glomerulonephritis, rheumatoid arthritis, multiple sclerosis, herpes simplex keratitis, and autoimmune disease.

13. A method of enhancing production of a type-1 immune response-associated cytokine by an immune cell comprising contacting said cell with an Eta-1/osteopontin stimulatory modulator such that production of the cytokine is enhanced.

14. The method of claim 13, wherein the type-1 immune response-associated cytokine is selected from the group consisting of interleukin-2 (IL-2), interleukin-12 (IL-12) and interferon-γ (IFN-γ).

15. A method of downregulating production of a type-2 immune response-associated cytokine by an immune cell comprising contacting said cell with an Eta-1/osteopontin inhibitory modulator such that production of the cytokine is downregulated.

16. The method of claim 15, wherein the type-2 immune response-associated cytokine is selected from the group consisting of interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-6 (IL-6), and interleukin-10 (IL-10).

17. The method of claim 13 or 15, wherein the cell is a human cell.

18. The method of claim 13, wherein the immune cell is contacted in vivo.

19. The method of claim 13, wherein the immune cell is contacted ex vivo.

20. The method of claim 13, wherein the immune cell is selected from the group consisting of a macrophage, a dendritic cell, a T cell, a B cell, a monocyte and a neutrophil.

21. A method for stimulating interleukin-12 (IL-12) production by a macrophage comprising contacting said macrophage with an Eta-1/osteopontin stimulatory modulator such that production of IL-12 is stimulated.

22. A method for inhibiting interleukin-10 (IL-10) production by a macrophage comprising contacting said macrophage with an Eta-1/osteopontin stimulatory modulator such that production of IL-10 is inhibited.

23. A method for potentiating a type-1 immune response in a subject comprising:

(a) culturing immune effector cells isolated from said subject in the presence of an Eta-1/osteopontin stimulatory modulator; and

(b) administering the cultured cells to said subject such that the type-1 immune response in said subject is potentiated.

24. Modified tumor cells comprising irradiated tumor cells transduced with Eta-1/osteopontin.

25. The modified tumor cells of claim 24, wherein the cells are further transduced with GMCSF.

26. The modified tumor cells of claim 24, further comprising a pharmaceutically acceptable carrier.

27. The method of claim 1, wherein said Eta-1/osteopontin modulator is selected from the group consisting of an isolated Eta-1/osteopontin polypeptide, a biologically active fragment of an Eta-1/osteopontin polypeptide, an isolated nucleic acid molecule which encodes an Eta-1/osteopontin polypeptide and an isolated nucleic acid molecule which encodes a biologically active fragment of an Eta-1/osteopontin polypeptide.

28. The method of claim 27, wherein said Eta-1/osteopontin modulator is an isolated Eta-1/osteopontin polypeptide or biologically active fragment thereof.

29. The method of claim 28, wherein said Eta-1/osteopontin polypeptide is a human Eta-1/osteopontin polypeptide.

30. The method of claim 28, wherein said Eta-1/osteopontin polypeptide is at least 90% identical to a polypeptide having the amino acid sequence of SEQ ID NO:2.

31. The method of claim 27, wherein said Eta-1/osteopontin polypeptide comprises an amino acid sequence selected from the group consisting of SEQ ID NO:2, SEQ ID NO:4, and SEQ ID NO:6.

32. The method of claim 27, wherein said Eta-1/osteopontin modulator is an isolated nucleic acid molecule encoding an Eta-1/osteopontin polypeptide or biologically active fragment thereof.

33. The method of claim 32, wherein said nucleic acid molecule is at least 90% identical to a nucleic acid molecule having the nucleotide sequence of SEQ ID NO:1.

34. The method of claim 32, wherein said nucleic acid molecule comprises a nucleotide sequence selected from the group consisting of SEQ ID NO:1, SEQ ID NO:3 and SEQ ID NO:5.

35. The method of claim 28, wherein the Eta-1/osteopontin modulator is a biologically active fragment of Eta-1/osteopontin.

36. The method of claim 32, wherein the Eta-1/osteopontin modulator is a nucleic acid molecule encoding a biologically active fragment of Eta-1/osteopontin.

37. The method of claim 35 or 36, wherein said biologically active fragment consists essentially of an IL-12 stimulatory domain of Eta-1/osteopontin.

38. The method of claim 37, wherein said IL-12 stimulatory domain comprises an amino acid sequence between 65 and 160 amino acids in length and is at least 90% identical to amino acids 71 to 168 of SEQ ID NO:2.

39. The method of claim 35 or 36, wherein said biologically active fragment consists essentially of an IL-10 inhibitory domain of Eta-1/osteopontin.

40. The method of claim 35, wherein said IL-10 inhibitory domain comprises an amino acid sequence between 65 and 160 amino acids in length and is at least 90% identical to amino acids 169-266 of SEQ ID NO:2.

41. The method of claim 1, wherein said Eta-1/osteopontin modulator is selected from the group consisting of a compound which specifically binds an Eta-1/osteopontin polypeptide, a compound which specifically binds an Eta-1/osteopontin target molecule, a compound which specifically modulates the activity of an Eta-1/osteopontin polypeptide and a compound which specifically modulates the activity of an Eta-1/osteopontin target molecule.

42. The method of claim 41, wherein said Eta-1/osteopontin modulator is an antibody which specifically binds Eta-1/osteopontin.

43. The method of claim 1, wherein said Eta-1/osteopontin modulator is a biosynthetic immunomodulatory molecule.

44. A biosynthetic immunomodulatory molecule comprising an IL-12 stimulatory component and a first biomodular component, forming a molecule which modulates an immune response.

45. The immunomodulatory molecule of claim 44, wherein the IL-12 stimulatory component is derived from Eta-1/osteopontin.

46. The immunomodulatory molecule of claim 45, wherein the IL-12 stimulatory component is a polypeptide.

47. The immunomodulatory molecule of claim 46, wherein the IL-12 stimulatory component comprises an amino acid sequence between 65 and 160 amino acid residues in length and is at least 90% identical to amino acids 71 to 168 of SEQ ID NO:2.

48. The immunomodulatory molecule of claim 46, wherein the IL-12 stimulatory component comprises amino acids 71-168 of SEQ ID NO:2.

49. A biosynthetic immunomodulatory molecule comprising an IL-10 inhibitory component and a first biomodular component, forming a molecule which modulates an immune response.

50. The immunomodulatory molecule of claim 49, wherein the IL-10 inhibitory component is derived from Eta-1/osteopontin.

51. The immunomodulatory molecule of claim 50, wherein the IL-10 inhibitory component is a polypeptide.

52. The immunomodulatory molecule of claim 50, wherein the IL-10 inhibitory component comprises an amino acid sequence between 65 and 160 amino acid residues in length and is at least 90% identical to amino acids 169 to 266 of SEQ ID NO:2.

53. The immunomodulatory molecule of claim 47, wherein the IL-10 inhibitory component comprises amino acids 169 to 266 of SEQ ID NO:2.

54. The immunomodulatory molecule of claim 44 or 49, wherein the first biomodular component is selected from the group consisting of a signal peptide, a calcium/apatite binding domain and a heparin binding domain.

55. The biosynthetic immunomodulatory molecule of claim 44 or 49, further comprising a second biomodular component.

56. The immunomodulatory molecule of claim 55, wherein the second biomodular component is selected from the group consisting of a signal peptide, a calcium/apatite binding domain and a heparin binding domain.

57. A biosynthetic immunomodulatory molecule comprising an IL-12 stimulatory component, a calcium/apatite binding domain and and a heparin binding domain.

58. A biosynthetic immunomodulatory molecule comprising an IL-10 inhibitory component, a signal peptide, a calcium/apatite binding domain and a heparin binding domain.

59. The immunomodulatory molecule of claim 44 or 49, wherein the molecule modulates an immune response selected from the group consisting of modulation of cytokine secretion, regulation of chemotaxis, regulation of hapotaxis, and regulation of cell spreading.

60. A biosynthetic immunomodulatory molecule comprising the amino acid sequence of SEQ ID NO:8.

61. A biosynthetic immunomodulatory molecule comprising the amino acid sequence of SEQ ID NO:10.

62. A biosynthetic immunomodulatory molecule encoded by nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO:7.

63. A biosynthetic immunomodulatory molecule encoded by nucleic acid molecule comprising the nucleotide sequence of SEQ ID NO:9.

64. An isolated nucleic acid molecule comprising nucleic acid sequences which encode the immunomodulatory molecule of claim 44 or 49.

65. An expression vector comprising the nucleic acid molecule of claim 64.

66. A host cell comprising the vector of claim 65.

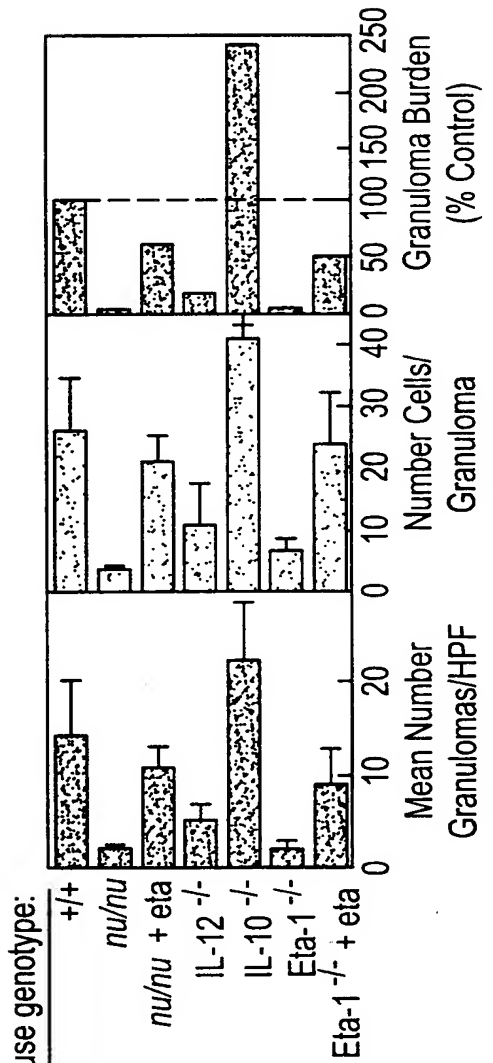
67. A method of producing an immunomodulatory molecule, comprising culturing the host cell of claim 66 under conditions such that the immunomodulatory molecule is produced.

68. A pharmaceutical composition comprising the immunomodulatory molecule of claim 44 or 49, and a pharmaceutically acceptable carrier.

69. A method of modulating an immune response in a cell comprising contacting the cell with an immunomodulatory molecule of claim 44 or 49 such that an immune response is modulated.

70. The method of claim 69, wherein the cell is present within a subject and the immunomodulatory molecule is administered to the subject.

FIG.1A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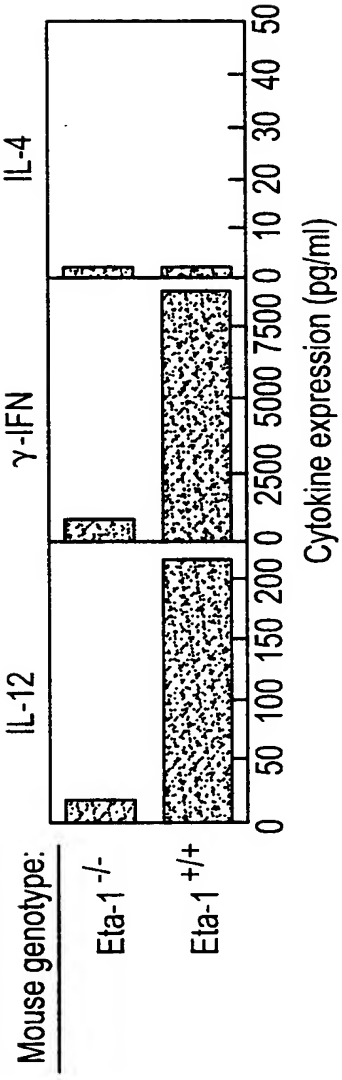
nu/nu
nu/nu + eta
IL-12 -/-
IL-10 -/-
Eta-1 -/-
Eta-1 -/- + eta
0 10 20
Mean Number Granulomas/HPF
0 10 20 30 40
Number Cells/ Granuloma
0 50 100 150 200 250
Granuloma Burden (% Control)

FIG.1C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Eta-1 +/+
IL-12
γ-IFN
IL-4
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0 2500 5000 7500
0 10 20 30 40 50
Cytokine expression (pg/ml)

FIG.1B

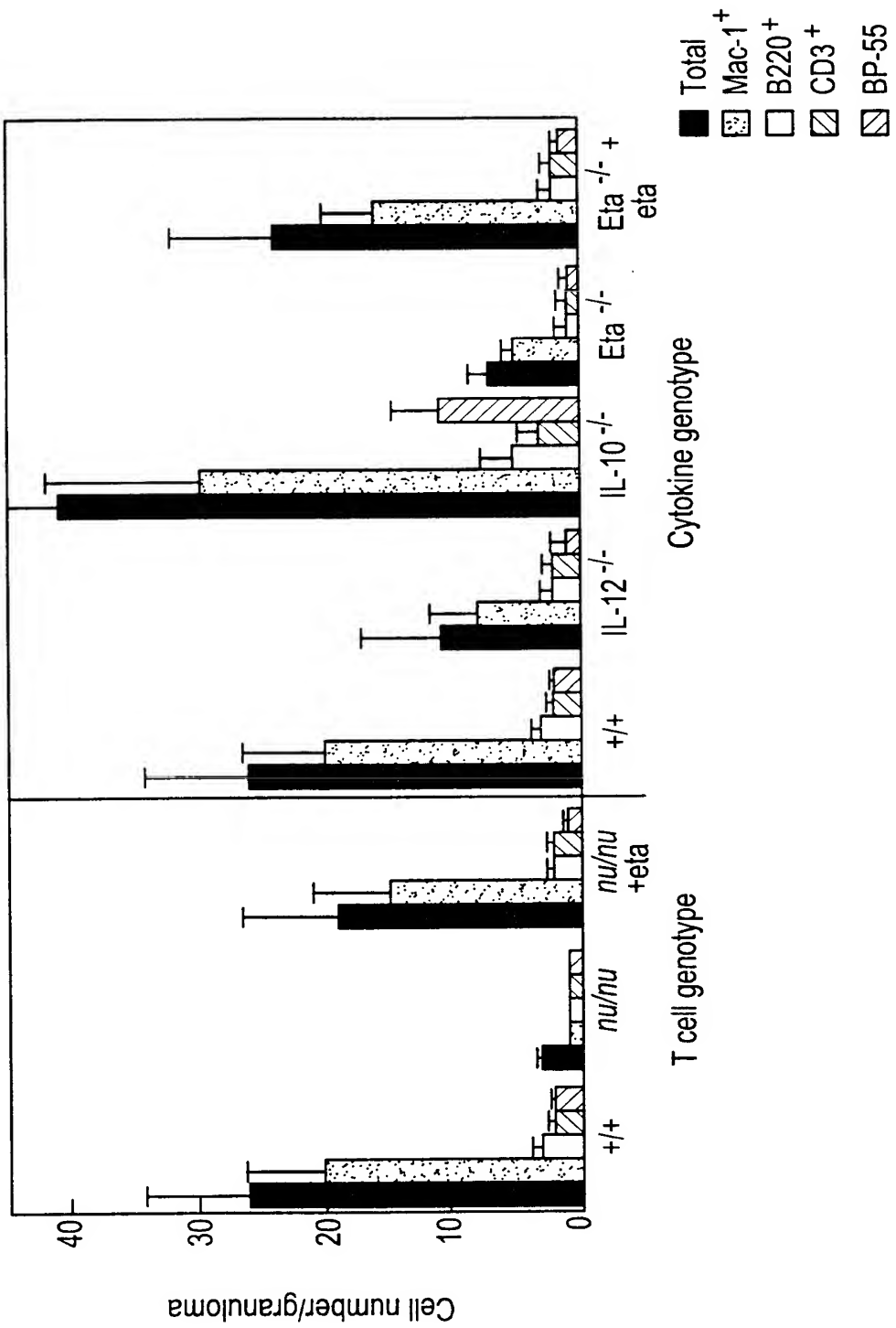

Cell number/granuloma
0
10
20
30
40
+/+
nu/nu
nu/nu +eta
+/+
IL-12 -/-
IL-10 -/-
Eta -/-
Eta -/- + eta
T cell genotype
Cytokine genotype
Total
Mac-1+
B220+
CD3+
BP-55

FIG. 2A

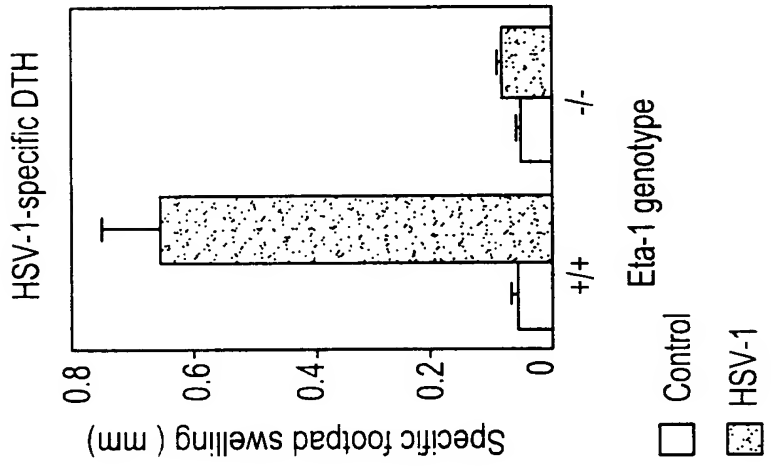

HSV-1-specific DTH
Specific footpad swelling (mm)
0
0.2
0.4
0.6
0.8
+/+
-/-
Eta-1 genotype
Control
HSV-1

FIG. 2B

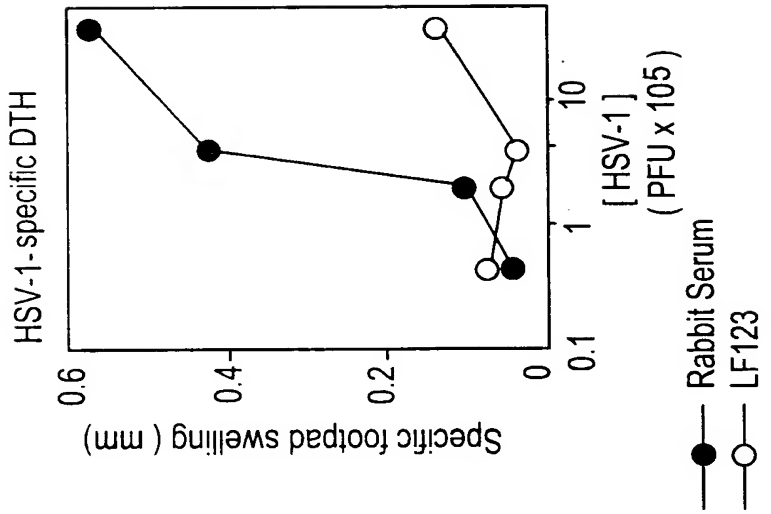

HSV-1-specific DTH
Specific footpad swelling (mm)
0
0.2
0.4
0.6
0.1
1
10
[HSV-1]
(PFU x 105)
Rabbit Serum
LF123

FIG. 2C

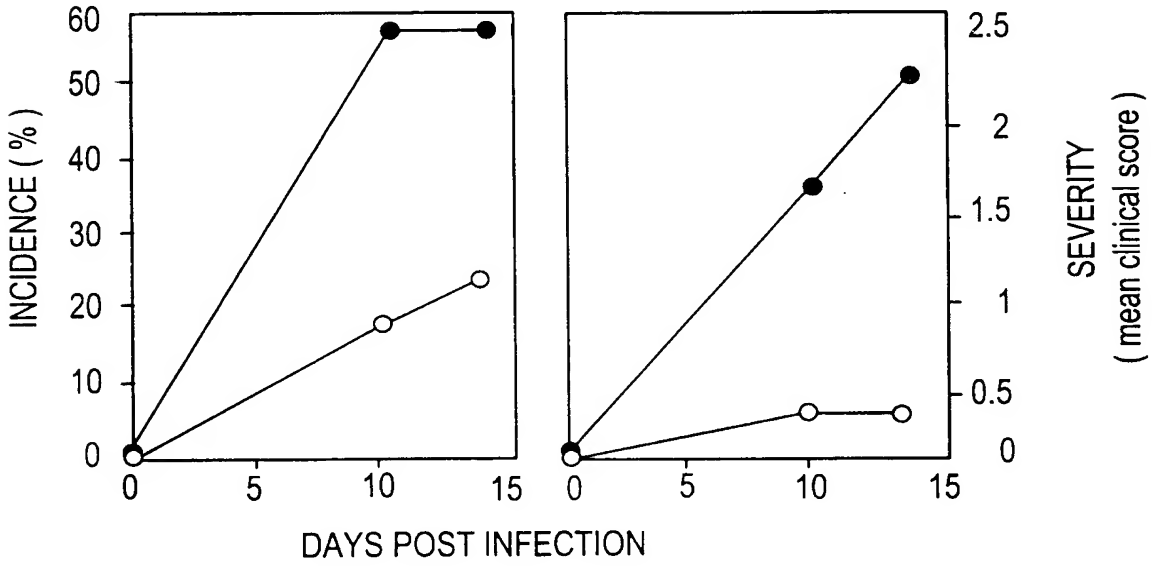

INCIDENCE (%)
60
50
40
30
20
10
0
0
5
10
15
2.5
2
1.5
1
0.5
0
SEVERITY
(mean clinical score)
DAYS POST INFECTION

FIG. 2D

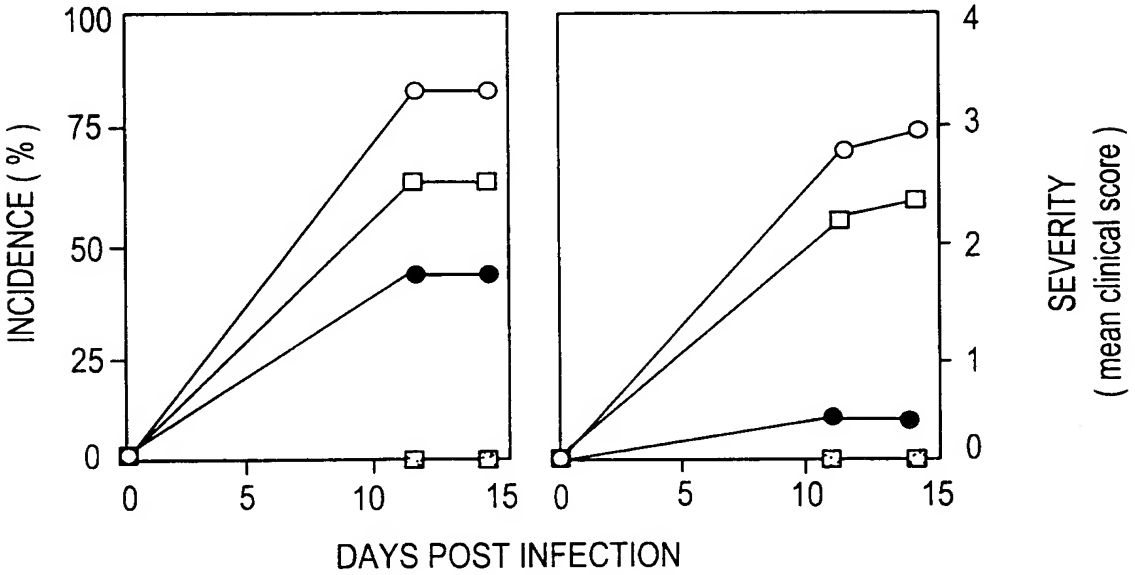

INCIDENCE (%)
100
75
50
25
0
0
5
10
15
4
3
2
1
0
SEVERITY
(mean clinical score)
DAYS POST INFECTION

FIG. 2E

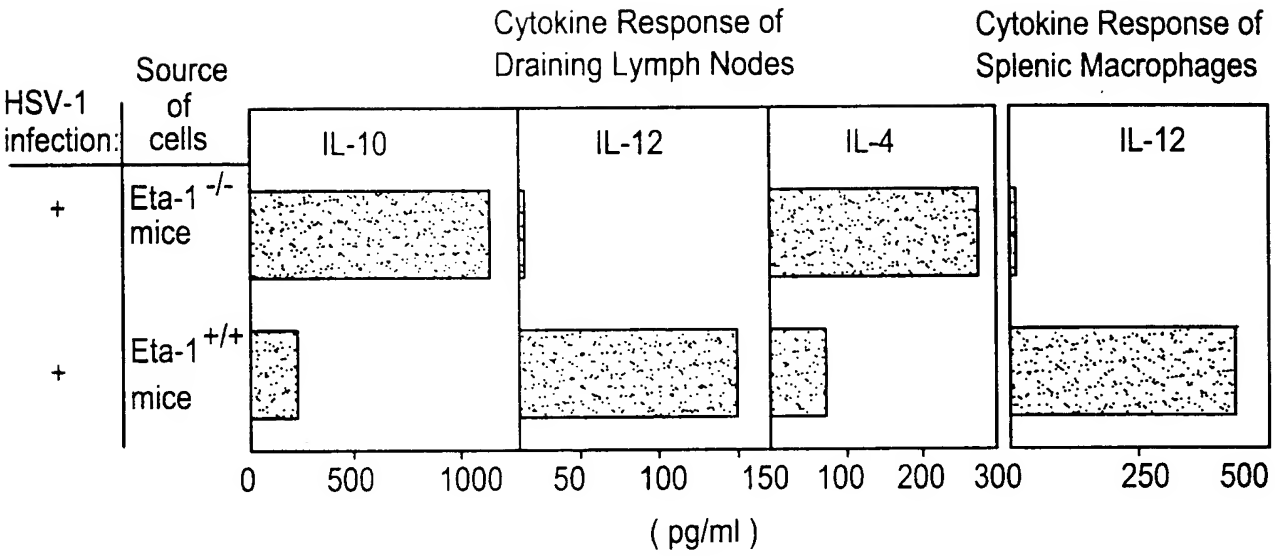

Cytokine Response of Draining Lymph Nodes
Cytokine Response of Splenic Macrophages
HSV-1 infection:
Source of cells
IL-10
IL-12
IL-4
IL-12
+
Eta-1 -/- mice
+
Eta-1 +/+ mice
0
500
1000
50
100
150
100
200
300
250
500
(pg/ml)

FIG. 3A

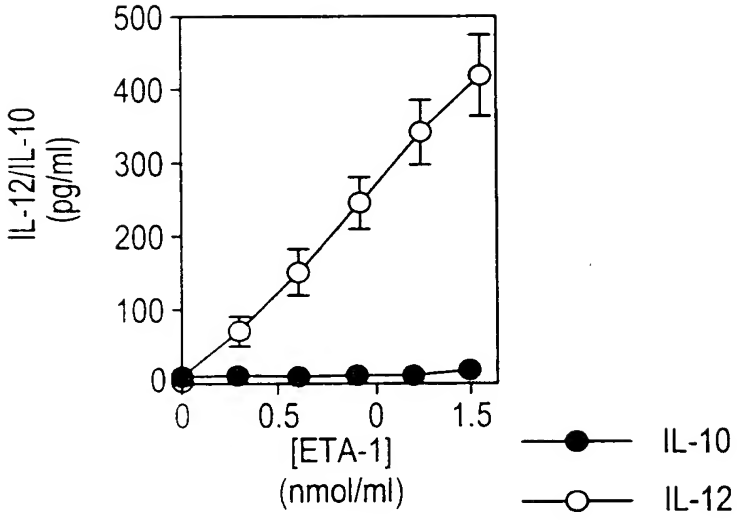

500
400
300
200
100
0
IL-12/IL-10
(pg/ml)
0
0.5
0
1.5
[ETA-1]
(nmol/ml)
IL-10
IL-12

FIG. 3B

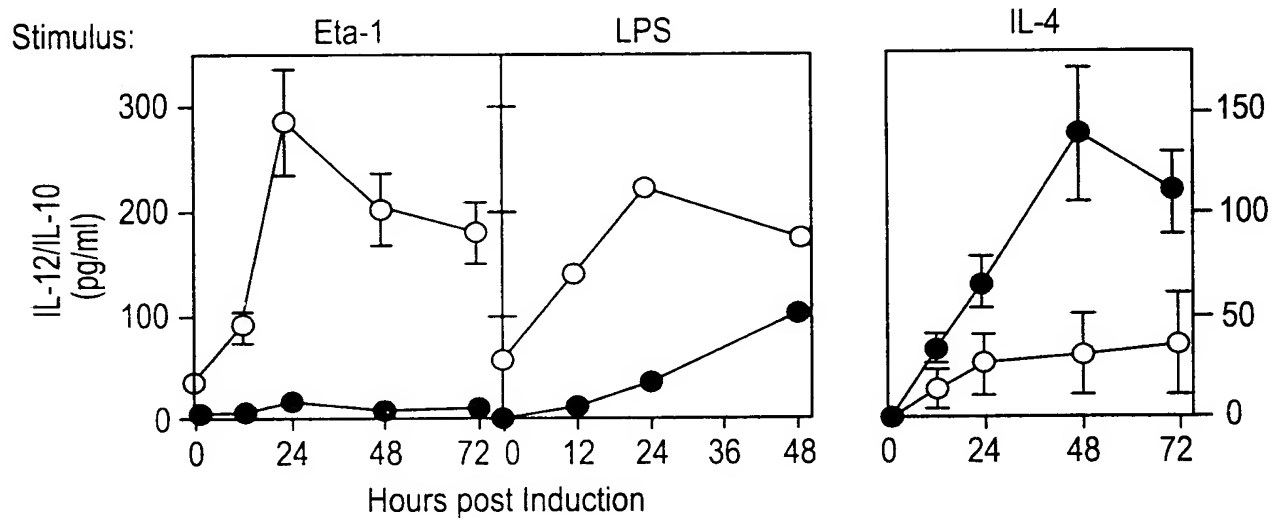

Stimulus:
Eta-1
LPS
IL-4
300
200
100
0
IL-12/IL-10
(pg/ml)
0
24
48
72
0
12
24
36
48
Hours post Induction
150
100
50
0
0
24
48
72

FIG. 3C

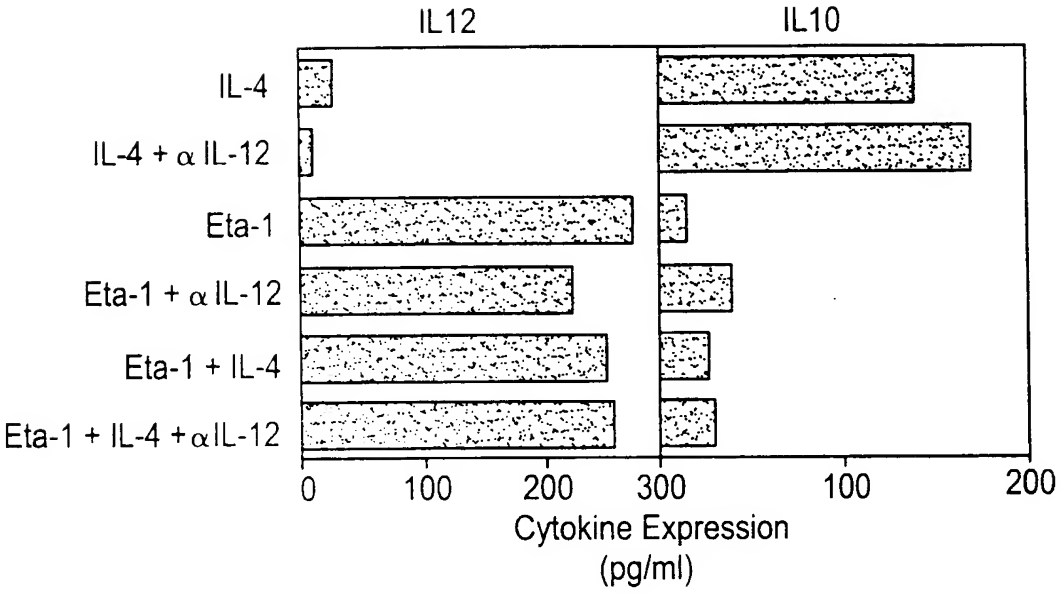

IL12
IL10
IL-4
IL-4 + α IL-12
Eta-1
Eta-1 + α IL-12
Eta-1 + IL-4
Eta-1 + IL-4 + α IL-12
0
100
200
300
100
200
Cytokine Expression
(pg/ml)

FIG. 3D

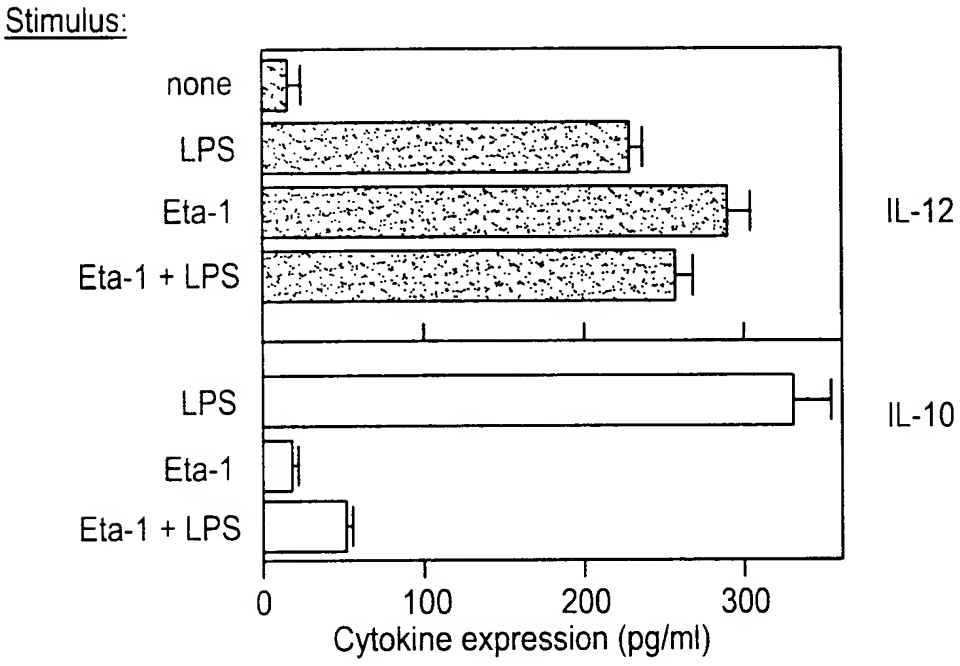

Stimulus:
none
LPS
Eta-1
Eta-1 + LPS
IL-12
LPS
Eta-1
Eta-1 + LPS
IL-10
0
100
200
300
Cytokine expression (pg/ml)

FIG. 4A

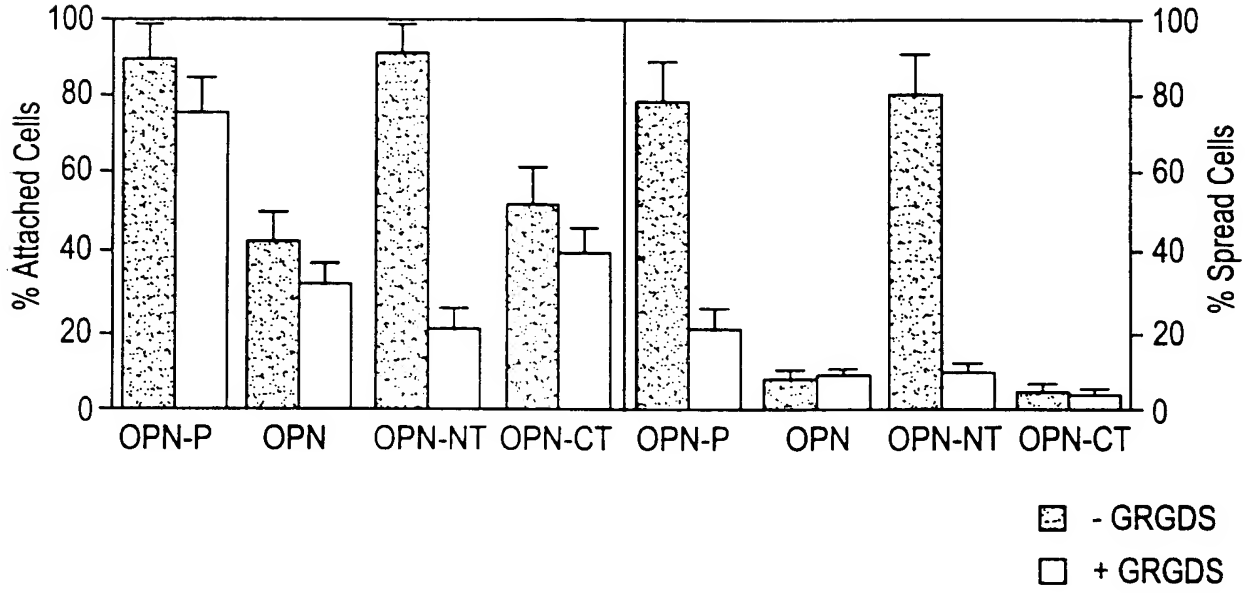

100
80
60
40
20
0
% Attached Cells
% Spread Cells
OPN-P
OPN
OPN-NT
OPN-CT
OPN-P
OPN
OPN-NT
OPN-CT
- GRGDS
+ GRGDS

FIG. 4B

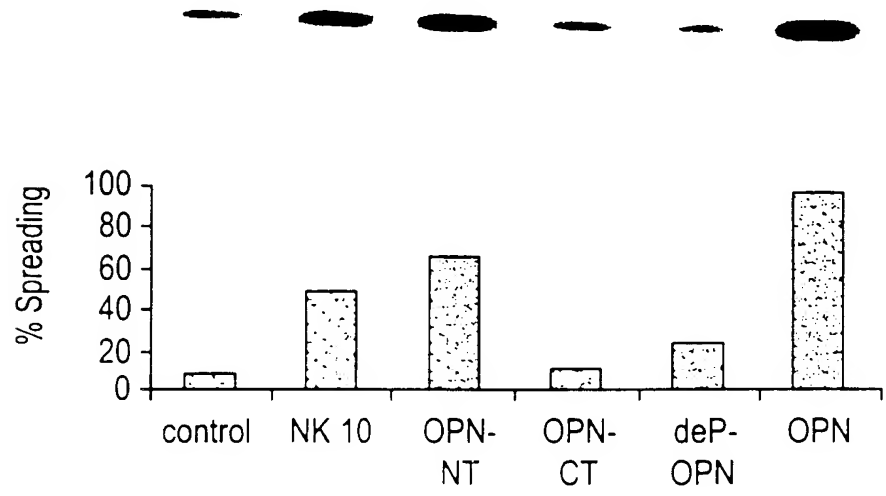

% Spreading
100
80
60
40
20
0
control
NK 10
OPN-NT
OPN-CT
deP-OPN
OPN

FIG.5A

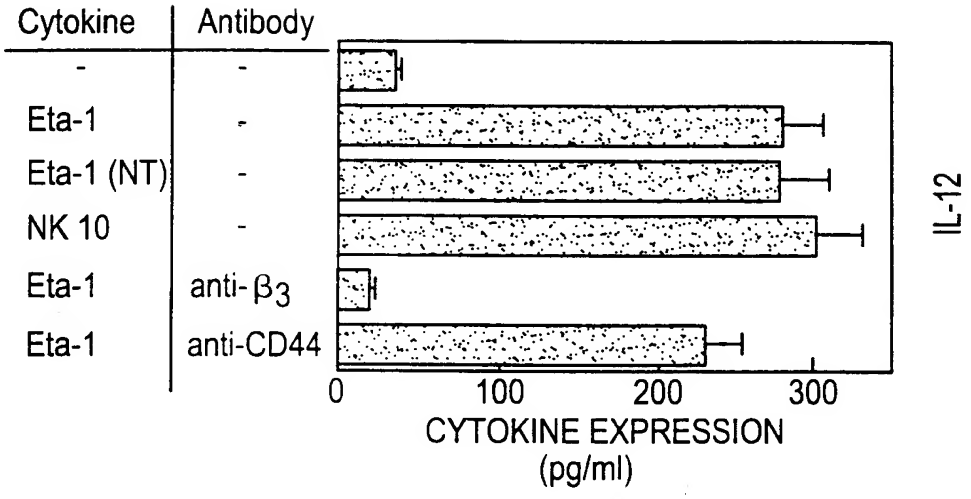


FIG.5B

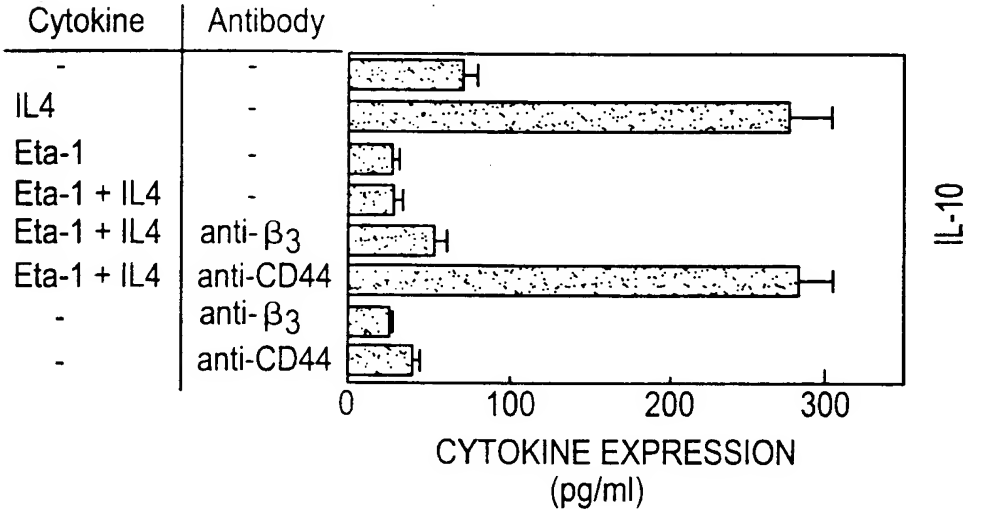


FIG.5C

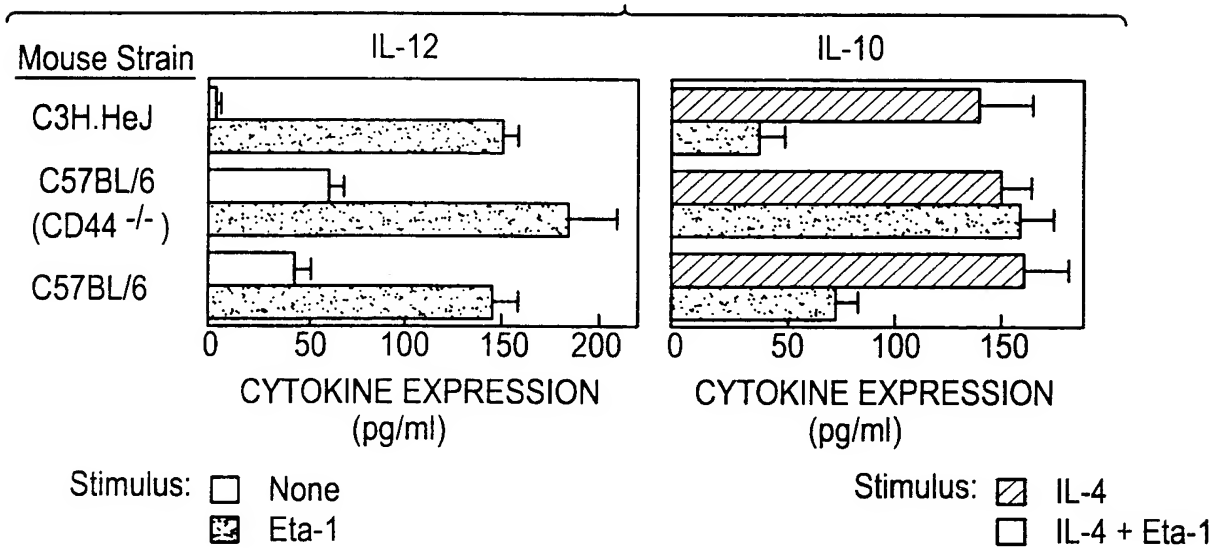


FIG.6A

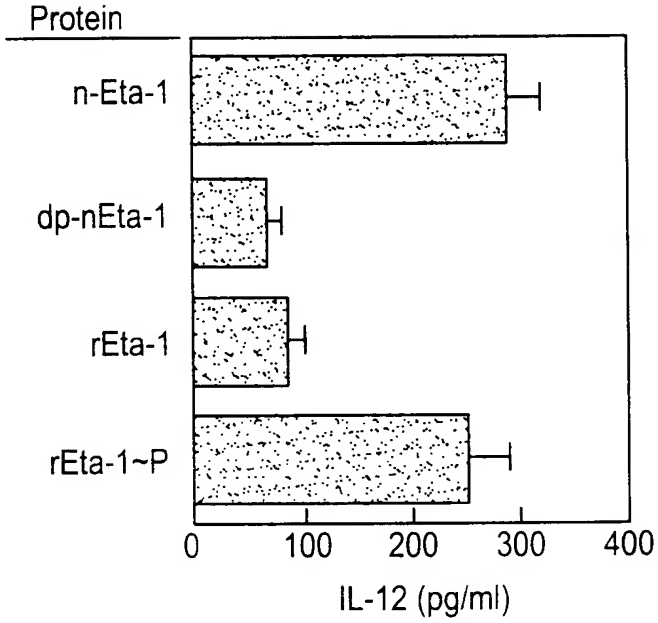

Protein
n-Eta-1
dp-nEta-1
rEta-1
rEta-1~P
0
100
200
300
400
IL-12 (pg/ml)

FIG.6B

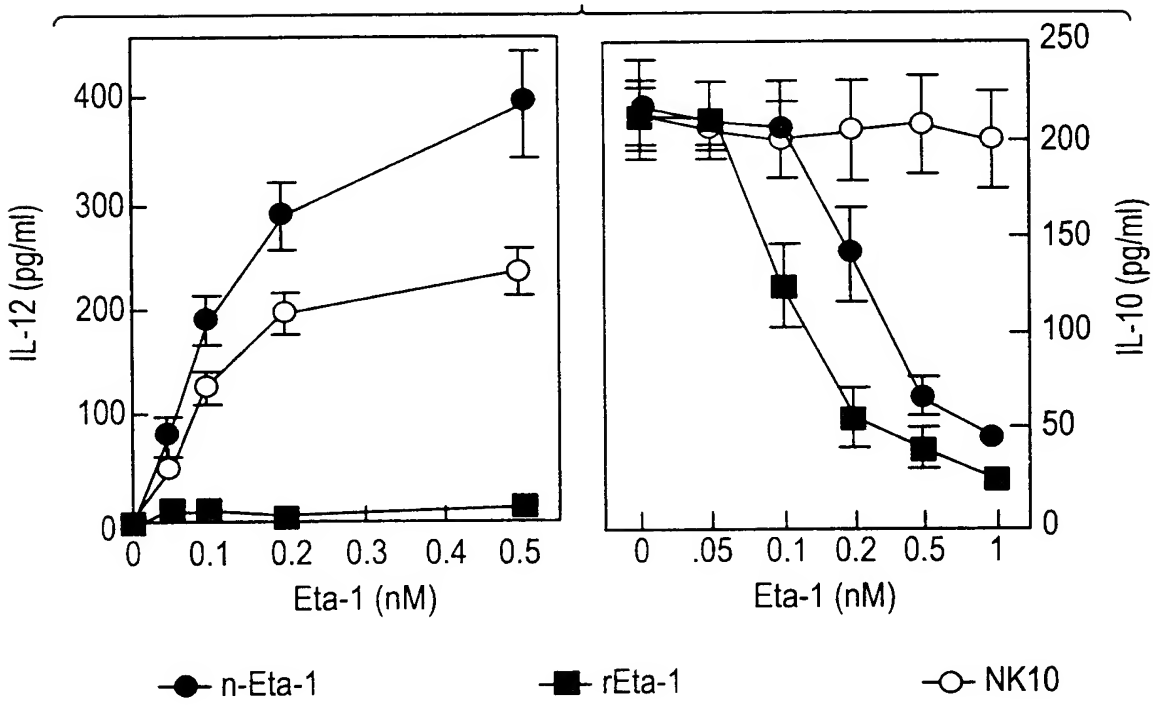

IL-12 (pg/ml)
0
100
200
300
400
0
0.1
0.2
0.3
0.4
0.5
Eta-1 (nM)
IL-10 (pg/ml)
0
50
100
150
200
250
0
.05
0.1
0.2
0.5
1
Eta-1 (nM)
n-Eta-1
rEta-1
NK10

FIG.7

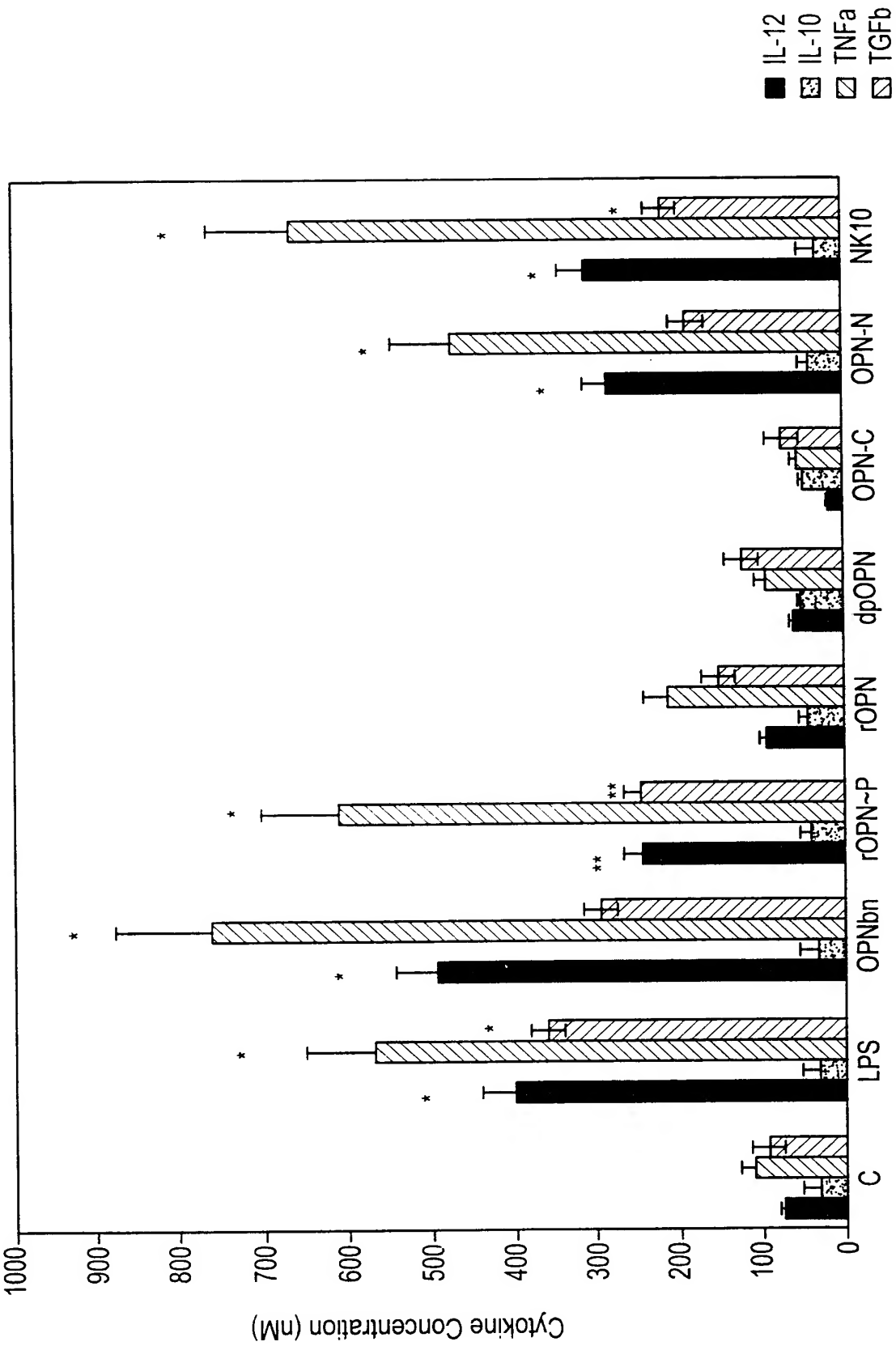

IL-12
IL-10
TNFa
TGFb
Cytokine Concentration (nM)
1000
900
800
700
600
500
400
300
200
100
0
C
LPS
OPNbn
rOPN~P
rOPN
dpOPN
OPN-C
OPN-N
NK10
*
**

FIG.8

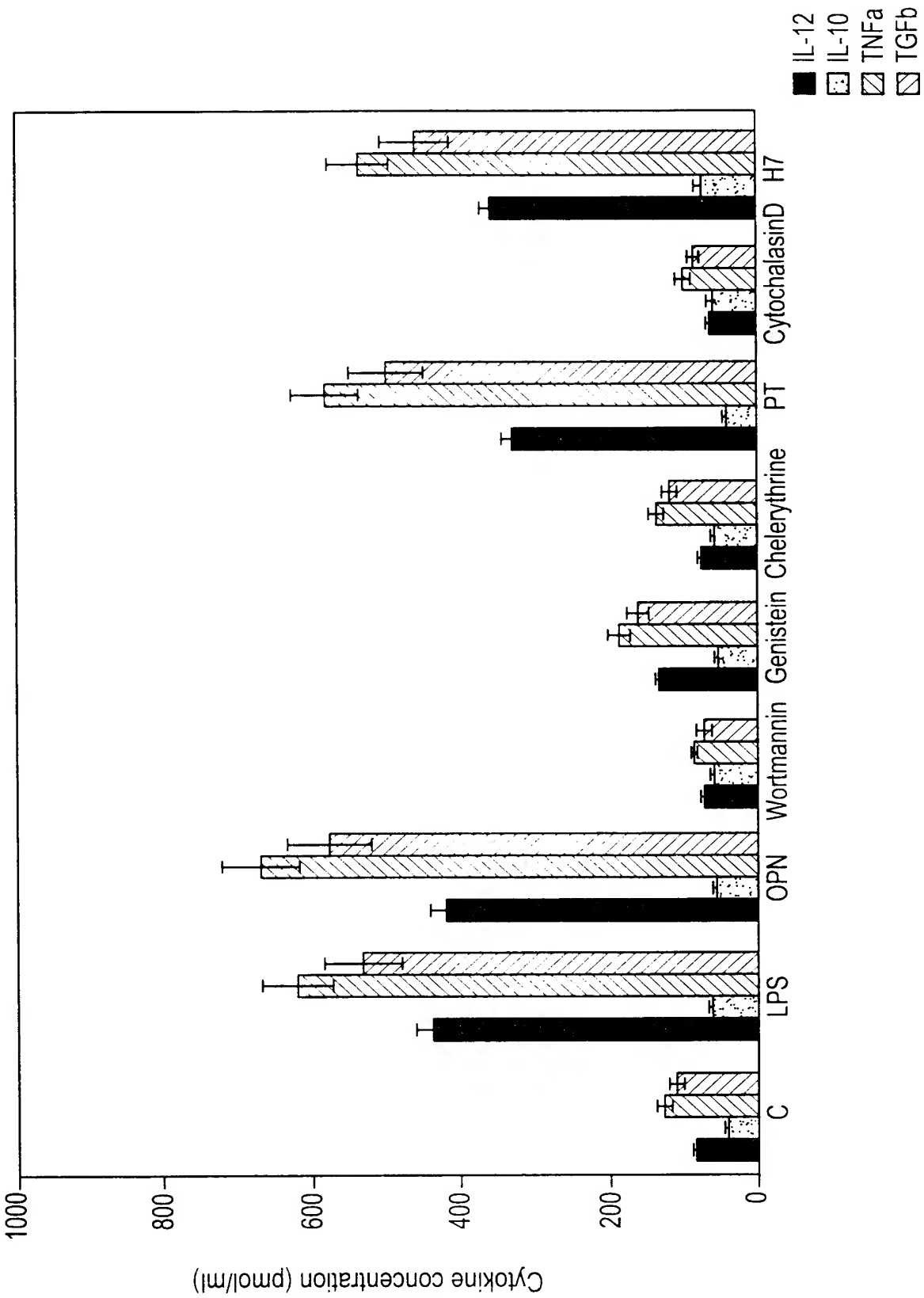

IL-12
IL-10
TNFa
TGFb
Cytokine concentration (pmol/ml)
0
200
400
600
800
1000
C
LPS
OPN
Wortmannin
Genistein
Chelerythrine
PT
CytochalasinD
H7

FIG.9

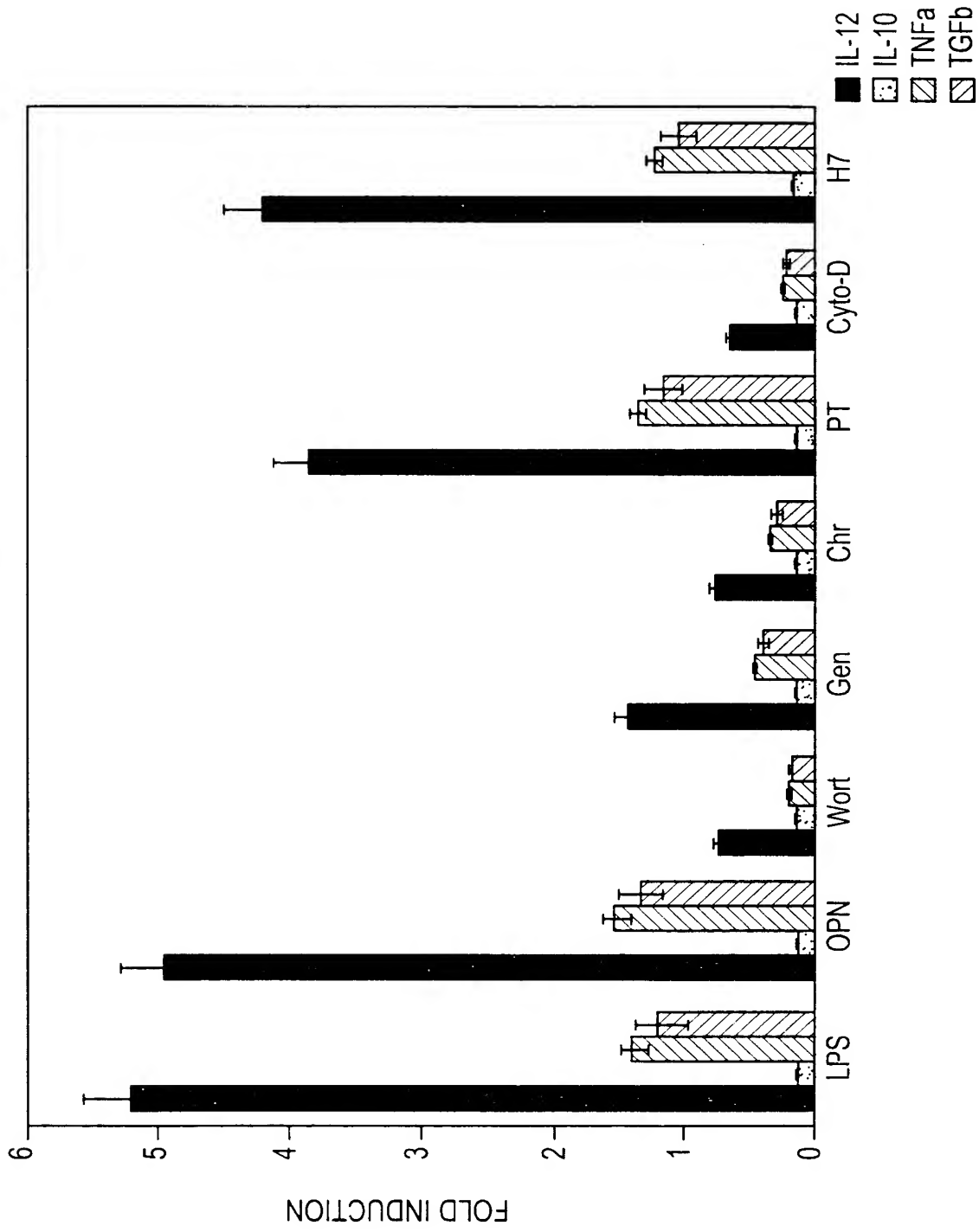

IL-12
IL-10
TNFa
TGFb
FOLD INDUCTION
0
1
2
3
4
5
6
LPS
OPN
Wort
Gen
Chr
PT
Cyto-D
H7

FIG. 10

```
                                agaattgcagtgatttgcttttgcctcctaggcatcacctgt
                                  R  I  A  V  I  C  F  C  L  L  G  I  T  C
gccataccagttaaacaggctgattctggaagttctgaggaattc
A  I  P  V  K  Q  A  D  S  G  G  S  S  E  E  F
Gatgatgaggagctctctcaccattctgatgaatctgatgaactggtcactgat
D  D  D  D  D  S  H  H  S  S  D  E  S  D  E  L  V  T  D

ttcccacggacctgccagcaaccgaagtttcactccagttgtc
F  P  T  D  L  P  A  T  E  V  F  T  P  V  V
cccacagtagacacatatgatggccgaggtgatagtgtggtttat
P  T  V  D  T  Y  D  G  R  G  D  S  V  V  Y
ggactgagtaa
G  L  R
```

FIG. 11

```
atgagaattgcagtgatttgcttttgcctcctaggcatcacctgt
 M  R  I  A  V  I  C  F  C  L  L  G  I  T  C
gccataccagttaaacaggctgattctggaagttctgaggaaatg
 A  I  P  V  K  Q  A  D  S  G  S  S  E  E  M
catcaccaccatcaccatctcaggtcaaaatctaagaagtttcgc
 H  H  H  H  H  H  L  R  S  K  S  K  K  F  R
agacctgacatccagtaccctgatgctacagacgaggacatcacc
 R  P  D  I  Q  Y  P  D  A  T  D  E  D  I  T
tcacacatggaaagcgaggagttgaatggtgcatacaaggccatc
 S  H  M  E  S  E  E  L  N  G  A  Y  K  A  I
cccgttgcccaggacctgaacgcgccttctgattgggacagccgt
 P  V  A  Q  D  L  N  A  P  S  D  W  D  S  R
gggaaggacagttatgaaacgagtcagctggatgaccagagtgct
 G  K  D  S  Y  E  T  S  Q  L  D  D  Q  S  A
gaaacccacagccacaagcagtccagattatataagcggaaagcc
 E  T  H  S  H  K  Q  S  R  L  Y  K  R  K  A
aatgatgagagcaatgagcattccgatgtgattgatagtcaggaa
 N  D  E  S  N  E  H  S  D  V  I  D  S  Q  E
ctttccaaagtcagccgtgaattccacagccatgaatttcacagc
 L  S  K  V  S  R  E  F  H  S  H  E  F  H  S
catgaagatatgctggttgtagaccccaaaagtaaggaagaagat
 H  E  D  M  L  V  V  D  P  K  S  K  E  E  D
aaacacctgaaatttcgtatttctcatgaattagatagtgcatct
 K  H  L  K  F  R  I  S  H  E  L  D  S  A  S
tctgaggtcaat
 S  E  V  N
```

FIG. 12

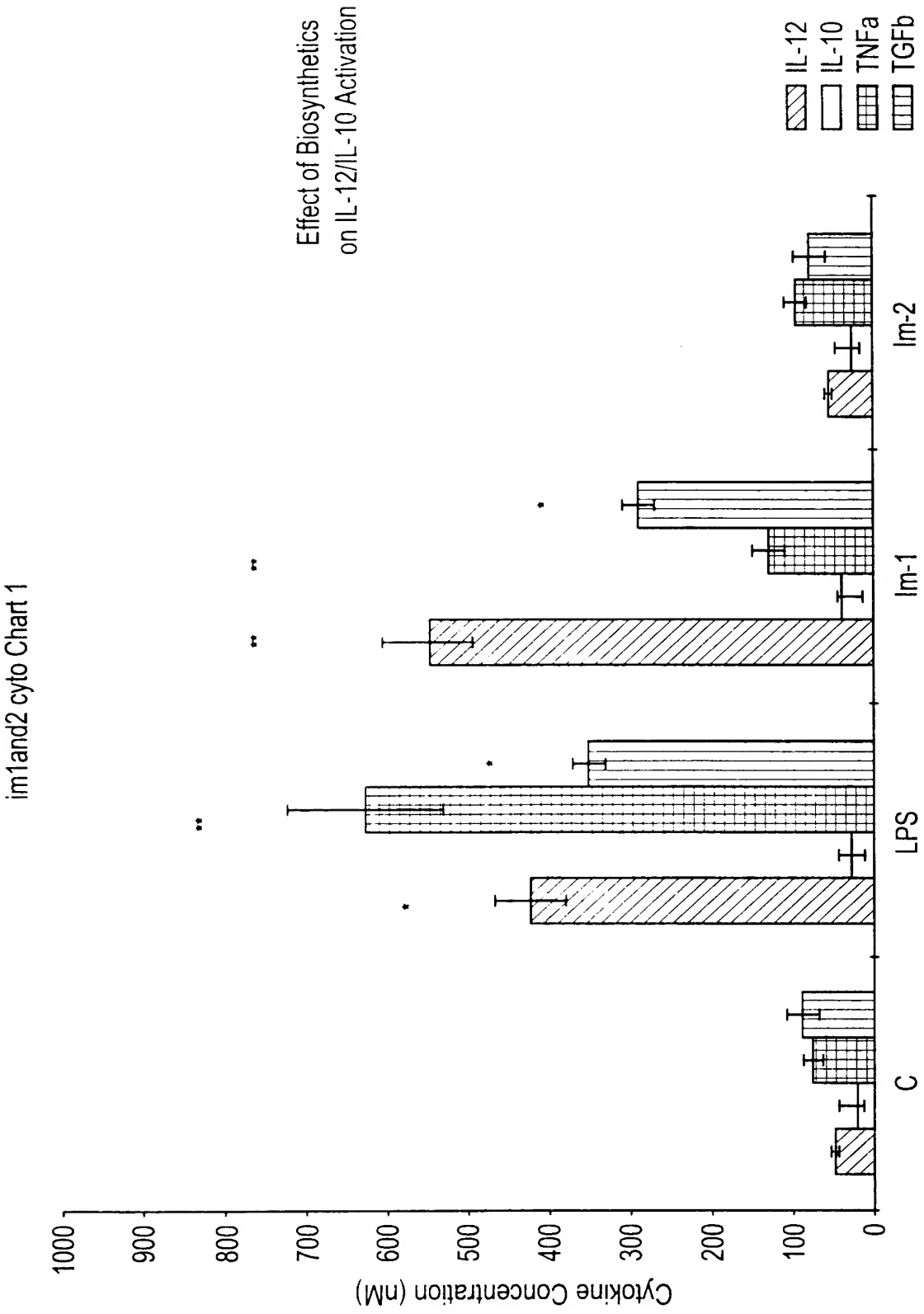

im1and2 cyto Chart 1
Effect of Biosynthetics
on IL-12/IL-10 Activation
IL-12
IL-10
TNFa
TGFb
Cytokine Concentration (nM)
0
100
200
300
400
500
600
700
800
900
1000
C
LPS
Im-1
Im-2

FIG. 13

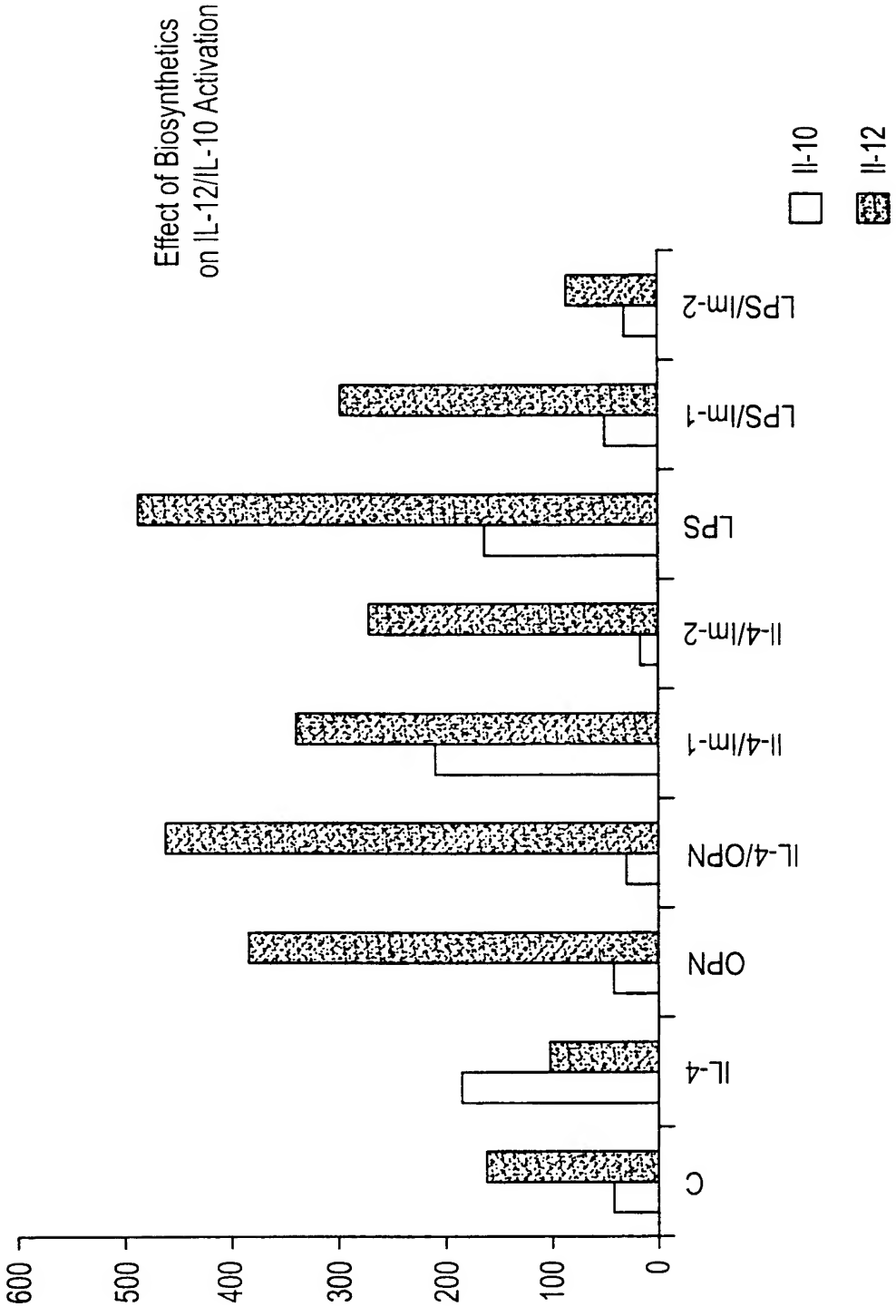
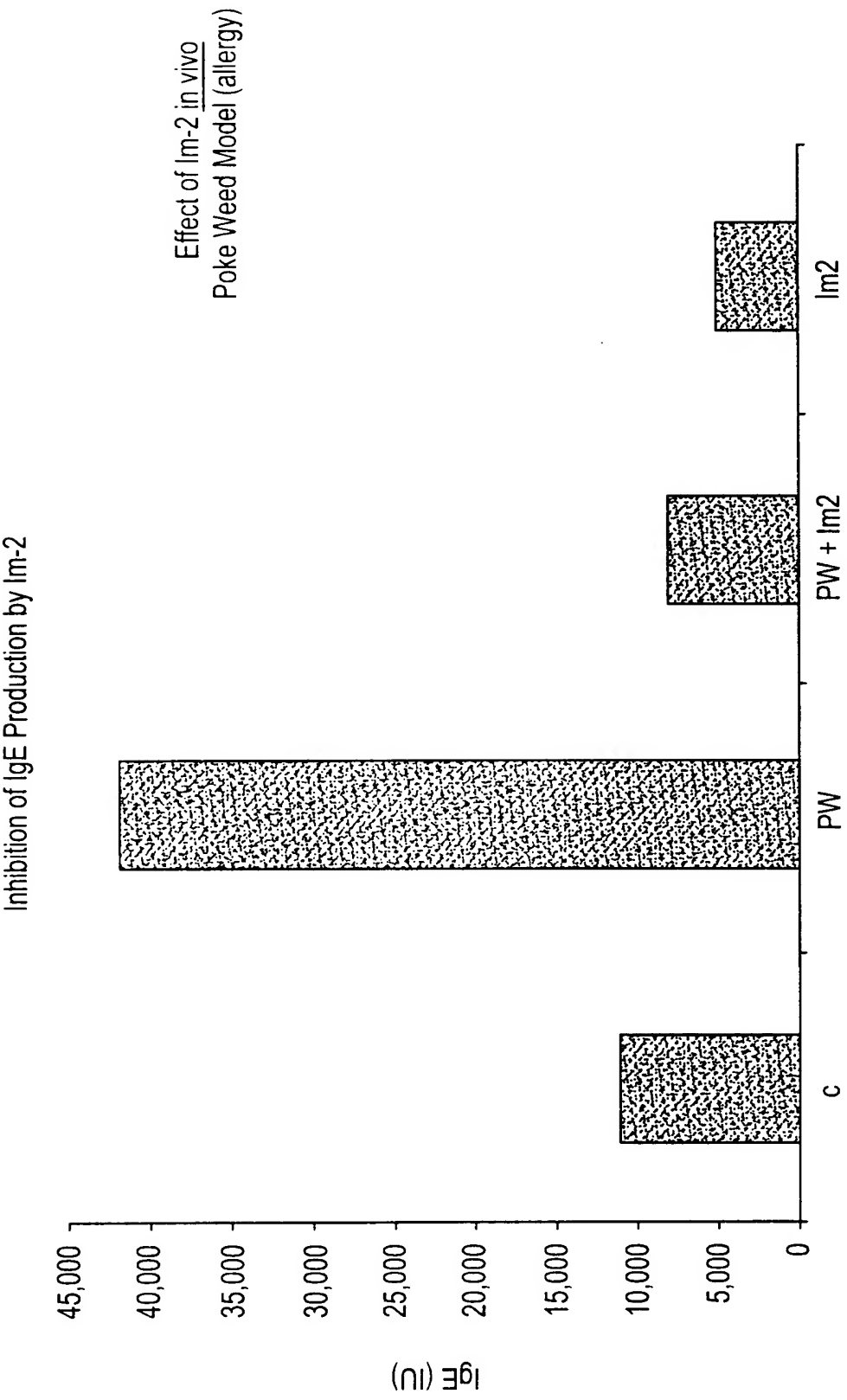

Effect of Biosynthetics on IL-12/IL-10 Activation
Il-10
Il-12
600
500
400
300
200
100
0
C
IL-4
OPN
IL-4/OPN
Il-4/Im-1
Il-4/Im-2
LPS
LPS/Im-1
LPS/Im-2

FIG. 14


Inhibition of IgE Production by Im-2
Effect of Im-2 in vivo
Poke Weed Model (allergy)
IgE (IU)
45,000
40,000
35,000
30,000
25,000
20,000
15,000
10,000
5,000
0
C
PW
PW + Im2
Im2

SEQUENCE LISTING

<110> DANA-FARBER CANCER INSTITUTE, INC.
CHILDREN'S MEDICAL CENTER CORPORATION

<120> METHODS AND COMPOSITIONS FOR MODULATING AN IMMUNE RESPONSE

<130> CMZ-121CPPC

<140>
<141>

<150> USSN 60/129,772
<151> 1999-04-15

<160> 6

<170> PatentIn Ver. 2.0

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<212> DNA
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<220>
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<222> (1)..(942)

<400> 1

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atg aga att gca gtg att tgc ttt tgc ctc cta ggc atc acc tgt gcc   48
Met Arg Ile Ala Val Ile Cys Phe Cys Leu Leu Gly Ile Thr Cys Ala
  1               5                  10                  15

ata cca gtt aaa cag gct gat tct gga agt tct gag gaa aag cag ctt   96
Ile Pro Val Lys Gln Ala Asp Ser Gly Ser Ser Glu Glu Lys Gln Leu
             20                  25                  30

tac aac aaa tac cca gat gct gtg gcc aca tgg cta aac cct gac cca   144
Tyr Asn Lys Tyr Pro Asp Ala Val Ala Thr Trp Leu Asn Pro Asp Pro
         35                  40                  45

tct cag aag cag aat ctc cta gcc cca cag aat gct gtg tcc tct gaa   192
Ser Gln Lys Gln Asn Leu Leu Ala Pro Gln Asn Ala Val Ser Ser Glu
     50                  55                  60

gaa acc aat gac ttt aaa caa gag acc ctt cca agt aag tcc aac gaa   240
Glu Thr Asn Asp Phe Lys Gln Glu Thr Leu Pro Ser Lys Ser Asn Glu
 65                  70                  75                  80

agc cat gac cac atg gat gat atg gat gat gaa gat gat gat gac cat   288
Ser His Asp His Met Asp Asp Met Asp Asp Glu Asp Asp Asp Asp His
                 85                  90                  95

gtg gac agc cag gac tcc att gac tcg aac gac tct gat gat gta gat   336
Val Asp Ser Gln Asp Ser Ile Asp Ser Asn Asp Ser Asp Asp Val Asp
            100                 105                 110
```

```
gac act gat gat tct cac cag tct gat gag tct cac cat tct gat gaa   384
Asp Thr Asp Asp Ser His Gln Ser Asp Glu Ser His His Ser Asp Glu
        115                 120                 125

tct gat gaa ctg gtc act gat ttt ccc acg gac ctg cca gca acc gaa   432
Ser Asp Glu Leu Val Thr Asp Phe Pro Thr Asp Leu Pro Ala Thr Glu
    130                 135                 140

gtt ttc act cca gtt gtc ccc aca gta gac aca tat gat ggc cga ggt   480
Val Phe Thr Pro Val Val Pro Thr Val Asp Thr Tyr Asp Gly Arg Gly
145                 150                 155                 160

gat agt gtg gtt tat gga ctg agg tca aaa tct aag aag ttt cgc aga   528
Asp Ser Val Val Tyr Gly Leu Arg Ser Lys Ser Lys Lys Phe Arg Arg
                165                 170                 175

cct gac atc cag tac cct gat gct aca gac gag gac atc acc tca cac   576
Pro Asp Ile Gln Tyr Pro Asp Ala Thr Asp Glu Asp Ile Thr Ser His
            180                 185                 190

atg gaa agc gag gag ttg aat ggt gca tac aag gcc atc ccc gtt gcc   624
Met Glu Ser Glu Glu Leu Asn Gly Ala Tyr Lys Ala Ile Pro Val Ala
        195                 200                 205

cag gac ctg aac gcg cct tct gat tgg gac agc cgt ggg aag gac agt   672
Gln Asp Leu Asn Ala Pro Ser Asp Trp Asp Ser Arg Gly Lys Asp Ser
    210                 215                 220

tat gaa acg agt cag ctg gat gac cag agt gct gaa acc cac agc cac   720
Tyr Glu Thr Ser Gln Leu Asp Asp Gln Ser Ala Glu Thr His Ser His
225                 230                 235                 240

aag cag tcc aga tta tat aag cgg aaa gcc aat gat gag agc aat gag   768
Lys Gln Ser Arg Leu Tyr Lys Arg Lys Ala Asn Asp Glu Ser Asn Glu
                245                 250                 255

cat tcc gat gtg att gat agt cag gaa ctt tcc aaa gtc agc cgt gaa   816
His Ser Asp Val Ile Asp Ser Gln Glu Leu Ser Lys Val Ser Arg Glu
            260                 265                 270

ttc cac agc cat gaa ttt cac agc cat gaa gat atg ctg gtt gta gac   864
Phe His Ser His Glu Phe His Ser His Glu Asp Met Leu Val Val Asp
        275                 280                 285

ccc aaa agt aag gaa gaa gat aaa cac ctg aaa ttt cgt att tct cat   912
Pro Lys Ser Lys Glu Glu Asp Lys His Leu Lys Phe Arg Ile Ser His
    290                 295                 300

gaa tta gat agt gca tct tct gag gtc aat taa                       945
Glu Leu Asp Ser Ala Ser Ser Glu Val Asn
305                 310
```

<210> 2
<211> 314
<212> PRT
<213> Homo sapiens

<400> 2

```
Met Arg Ile Ala Val Ile Cys Phe Cys Leu Leu Gly Ile Thr Cys Ala
 1               5                  10                  15

Ile Pro Val Lys Gln Ala Asp Ser Gly Ser Ser Glu Glu Lys Gln Leu
            20                  25                  30

Tyr Asn Lys Tyr Pro Asp Ala Val Ala Thr Trp Leu Asn Pro Asp Pro
        35                  40                  45

Ser Gln Lys Gln Asn Leu Leu Ala Pro Gln Asn Ala Val Ser Ser Glu
    50                  55                  60

Glu Thr Asn Asp Phe Lys Gln Glu Thr Leu Pro Ser Lys Ser Asn Glu
65                  70                  75                  80

Ser His Asp His Met Asp Asp Met Asp Asp Glu Asp Asp Asp Asp His
                85                  90                  95

Val Asp Ser Gln Asp Ser Ile Asp Ser Asn Asp Ser Asp Asp Val Asp
            100                 105                 110

Asp Thr Asp Asp Ser His Gln Ser Asp Glu Ser His His Ser Asp Glu
        115                 120                 125

Ser Asp Glu Leu Val Thr Asp Phe Pro Thr Asp Leu Pro Ala Thr Glu
    130                 135                 140

Val Phe Thr Pro Val Val Pro Thr Val Asp Thr Tyr Asp Gly Arg Gly
145                 150                 155                 160

Asp Ser Val Val Tyr Gly Leu Arg Ser Lys Ser Lys Lys Phe Arg Arg
                165                 170                 175

Pro Asp Ile Gln Tyr Pro Asp Ala Thr Asp Glu Asp Ile Thr Ser His
            180                 185                 190

Met Glu Ser Glu Glu Leu Asn Gly Ala Tyr Lys Ala Ile Pro Val Ala
        195                 200                 205

Gln Asp Leu Asn Ala Pro Ser Asp Trp Asp Ser Arg Gly Lys Asp Ser
    210                 215                 220

Tyr Glu Thr Ser Gln Leu Asp Asp Gln Ser Ala Glu Thr His Ser His
225                 230                 235                 240

Lys Gln Ser Arg Leu Tyr Lys Arg Lys Ala Asn Asp Glu Ser Asn Glu
                245                 250                 255

His Ser Asp Val Ile Asp Ser Gln Glu Leu Ser Lys Val Ser Arg Glu
            260                 265                 270

Phe His Ser His Glu Phe His Ser His Glu Asp Met Leu Val Val Asp
        275                 280                 285

Pro Lys Ser Lys Glu Glu Asp Lys His Leu Lys Phe Arg Ile Ser His
    290                 295                 300
```

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Ile Pro Val Lys Gln Ala Asp Ser Gly Ser Ser Glu Glu Lys Gln Leu
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tac aac aaa tac cca gat gct gtg gcc aca tgg cta aac cct gac cca   144
Tyr Asn Lys Tyr Pro Asp Ala Val Ala Thr Trp Leu Asn Pro Asp Pro
         35                  40                  45

tct cag aag cag aat ctc cta gcc cca cag acc ctt cca agt aag tcc   192
Ser Gln Lys Gln Asn Leu Leu Ala Pro Gln Thr Leu Pro Ser Lys Ser
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aac gaa agc cat gac cac atg gat gat atg gat gat gaa gat gat gat   240
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 65                  70                  75                  80

gac cat gtg gac agc cag gac tcc att gac tcg aac gac tct gat gat   288
Asp His Val Asp Ser Gln Asp Ser Ile Asp Ser Asn Asp Ser Asp Asp
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gta gat gac act gat gat tct cac cag tct gat gag tct cac cat tct   336
Val Asp Asp Thr Asp Asp Ser His Gln Ser Asp Glu Ser His His Ser
            100                 105                 110

gat gaa tct gat gaa ctg gtc act gat ttt ccc acg gac ctg cca gca   384
Asp Glu Ser Asp Glu Leu Val Thr Asp Phe Pro Thr Asp Leu Pro Ala
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Thr Glu Val Phe Thr Pro Val Val Pro Thr Val Asp Thr Tyr Asp Gly
    130                 135                 140

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aat gag cat tcc gat gtg att gat agt cag gaa ctt tcc aaa gtc agc   768
Asn Glu His Ser Asp Val Ile Asp Ser Gln Glu Leu Ser Lys Val Ser
                245                 250                 255

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Tyr Asn Lys Tyr Pro Asp Ala Val Ala Thr Trp Leu Asn Pro Asp Pro
         35                  40                  45

Ser Gln Lys Gln Asn Leu Leu Ala Pro Gln Thr Leu Pro Ser Lys Ser
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Asp His Val Asp Ser Gln Asp Ser Ile Asp Ser Asn Asp Ser Asp Asp
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Val Asp Asp Thr Asp Asp Ser His Gln Ser Asp Glu Ser His His Ser
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Asp Glu Ser Asp Glu Leu Val Thr Asp Phe Pro Thr Asp Leu Pro Ala
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Thr Glu Val Phe Thr Pro Val Val Pro Thr Val Asp Thr Tyr Asp Gly
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Arg Gly Asp Ser Val Val Tyr Gly Leu Arg Ser Lys Ser Lys Lys Phe
145                 150                 155                 160

Arg Arg Pro Asp Ile Gln Tyr Pro Asp Ala Thr Asp Glu Asp Ile Thr
                165                 170                 175

Ser His Met Glu Ser Glu Glu Leu Asn Gly Ala Tyr Lys Ala Ile Pro
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Val Ala Gln Asp Leu Asn Ala Pro Ser Asp Trp Asp Ser Arg Gly Lys
        195                 200                 205

Asp Ser Tyr Glu Thr Ser Gln Leu Asp Asp Gln Ser Ala Glu Thr His
    210                 215                 220

Ser His Lys Gln Ser Arg Leu Tyr Lys Arg Lys Ala Asn Asp Glu Ser
225                 230                 235                 240

Asn Glu His Ser Asp Val Ile Asp Ser Gln Glu Leu Ser Lys Val Ser
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Arg Glu Phe His Ser His Glu Phe His Ser His Glu Asp Met Leu Val
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Ile Pro Val Lys Gln Ala Asp Ser Gly Ser Ser Glu Glu Lys Gln Asn
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gct gtg tcc tct gaa gaa acc aat gac ttt aaa caa gag acc ctt cca   144
Ala Val Ser Ser Glu Glu Thr Asn Asp Phe Lys Gln Glu Thr Leu Pro
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agt aag tcc aac gaa agc cat gac cac atg gat gat atg gat gat gaa   192
Ser Lys Ser Asn Glu Ser His Asp His Met Asp Asp Met Asp Asp Glu
     50                  55                  60

gat gat gat gac cat gtg gac agc cag gac tcc att gac tcg aac gac   240
Asp Asp Asp Asp His Val Asp Ser Gln Asp Ser Ile Asp Ser Asn Asp
 65                  70                  75                  80

tct gat gat gta gat gac act gat gat tct cac cag tct gat gag tct   288
Ser Asp Asp Val Asp Asp Thr Asp Asp Ser His Gln Ser Asp Glu Ser
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cac cat tct gat gaa tct gat gaa ctg gtc act gat ttt ccc acg gac   336
His His Ser Asp Glu Ser Asp Glu Leu Val Thr Asp Phe Pro Thr Asp
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ctg cca gca acc gaa gtt ttc act cca gtt gtc ccc aca gta gac aca   384
Leu Pro Ala Thr Glu Val Phe Thr Pro Val Val Pro Thr Val Asp Thr
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tat gat ggc cga ggt gat agt gtg gtt tat gga ctg agg tca aaa tct   432
Tyr Asp Gly Arg Gly Asp Ser Val Val Tyr Gly Leu Arg Ser Lys Ser
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aag aag ttt cgc aga cct gac atc cag tac cct gat gct aca gac gag   480
Lys Lys Phe Arg Arg Pro Asp Ile Gln Tyr Pro Asp Ala Thr Asp Glu
145                 150                 155                 160

gac atc acc tca cac atg gaa agc gag gag ttg aat ggt gca tac aag   528
Asp Ile Thr Ser His Met Glu Ser Glu Glu Leu Asn Gly Ala Tyr Lys
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gcc atc ccc gtt gcc cag gac ctg aac gcg cct tct gat tgg gac agc   576
Ala Ile Pro Val Ala Gln Asp Leu Asn Ala Pro Ser Asp Trp Asp Ser
            180                 185                 190

cgt ggg aag gac agt tat gaa acg agt cag ctg gat gac cag agt gct   624
Arg Gly Lys Asp Ser Tyr Glu Thr Ser Gln Leu Asp Asp Gln Ser Ala
        195                 200                 205

gaa acc cac agc cac aag cag tcc aga tta tat aag cgg aaa gct aat   672
Glu Thr His Ser His Lys Gln Ser Arg Leu Tyr Lys Arg Lys Ala Asn
    210                 215                 220

gat gag agc aat gag cat tcc gat gtg att gat agt cag gaa ctt tcc   720
Asp Glu Ser Asn Glu His Ser Asp Val Ile Asp Ser Gln Glu Leu Ser
225                 230                 235                 240

aaa gtc agc cgt gaa ttc cac agc cat gaa ttt cac agc cat gaa gat   768
Lys Val Ser Arg Glu Phe His Ser His Glu Phe His Ser His Glu Asp
                245                 250                 255
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atg ctg gtt gta gac ccc aaa agt aag gaa gaa gat aaa cac ctg aaa   816
Met Leu Val Val Asp Pro Lys Ser Lys Glu Glu Asp Lys His Leu Lys
            260                 265                 270

ttt cgt att tct cat gaa tta gat agt gca tct tct gag gtc aat taa   864
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        35                  40                  45

Ser Lys Ser Asn Glu Ser His Asp His Met Asp Asp Met Asp Asp Glu
    50                  55                  60

Asp Asp Asp Asp His Val Asp Ser Gln Asp Ser Ile Asp Ser Asn Asp
65                  70                  75                  80

Ser Asp Asp Val Asp Asp Thr Asp Asp Ser His Gln Ser Asp Glu Ser
                85                  90                  95

His His Ser Asp Glu Ser Asp Glu Leu Val Thr Asp Phe Pro Thr Asp
            100                 105                 110

Leu Pro Ala Thr Glu Val Phe Thr Pro Val Val Pro Thr Val Asp Thr
        115                 120                 125

Tyr Asp Gly Arg Gly Asp Ser Val Val Tyr Gly Leu Arg Ser Lys Ser
    130                 135                 140

Lys Lys Phe Arg Arg Pro Asp Ile Gln Tyr Pro Asp Ala Thr Asp Glu
145                 150                 155                 160

His Ile Thr Ser His Met Glu Ser Glu Glu Leu Asn Gly Ala Tyr Lys
                165                 170                 175

Ala Ile Pro Val Ala Gln Asp Leu Asn Ala Pro Ser Asp Trp Asp Ser
            180                 185                 190

Arg Gly Lys Asp Ser Tyr Glu Thr Ser Gln Leu Asp Asp Gln Ser Ala
        195                 200                 205

Glu Ala His Ser His Lys Gln Ser Arg Leu Tyr Lys Arg Lys Ala Asn
    210                 215                 220
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Asp Glu Ser Asn Glu His Ser Asp Val Ile Asp Ser Gln Glu Leu Ser
225                 230                 235                 240

Lys Val Ser Arg Glu Phe His Ser His Glu Phe His Ser His Glu Asp
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Met Leu Val Val Asp Pro Lys Ser Lys Glu Glu Asp Lys His Leu Lys
            260                 265                 270

Phe Arg Ile Ser His Glu Leu Asp Ser Ala Ser Ser Glu Val Asn
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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/10340

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K38/17 A61K48/00 C07K14/47 C12N5/10 C12N15/12
G01N33/50 A61P37/02

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 A61K C07K C12N G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
MEDLINE, BIOSIS, EPO-Internal, PAJ, WPI Data, LIFESCIENCES, CANCERLIT, STRAND

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 56405 A (WHITEHEAD INSTITUTE FOR BIOMEDICAL RESEARCH) 17 December 1998 (1998-12-17) page 23 -page 27 ---	1-13,23, 24,28, 29,32, 35,36, 41-43
X	YU X Q ET AL: "A functional role for osteopontin in experimental crescentic glomerulonephritis in the rat." PROCEEDINGS OF THE ASSOCIATION OF AMERICAN PHYSICIANS, (1998 JAN-FEB) 110 (1) 50-64. , XP000982503 the whole document --- -/--	1-13,23, 24,28, 29,32, 35,36, 41-43

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 February 2001	08/03/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31–70) 340–2040, Tx. 31 651 epo nl, Fax: (+31–70) 340–3016	Moreau, J

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

In ational Application No
PCT/US 00/10340

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	WEBER GEORG F ET AL: "The immunology of Eta-1/Osteopontin." CYTOKINE & GROWTH FACTOR REVIEWS, vol. 7, no. 3, 1996, pages 241-248, XP000982501 ISSN: 1359-6101 the whole document	1-70
P,X	ASHKAR SAMY ET AL: "Eta-1 (osteopontin): An early component of type-1 (cell-mediated) immunity." SCIENCE (WASHINGTON D C)., vol. 287, no. 5454, 4 February 2000 (2000-02-04), pages 860-864, XP002161278 ISSN: 0036-8075 the whole document	1-70

INTERNATIONAL SEARCH REPORT

Information on patent family members

In ational Application No
PCT/US 00/10340

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9856405 A	17-12-1998	NONE	